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The circadian rhythm of 5-hydroxytryptamine.

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'The Circadian Rhythm

of 5-Hydroxytryptamine'

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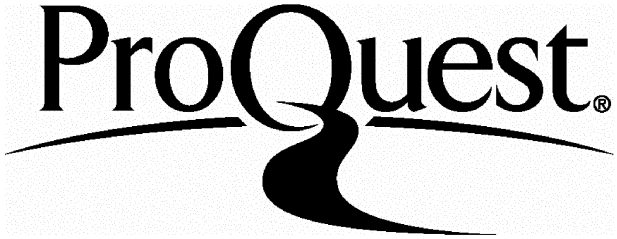
Jane Nicholass



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That period of twenty-four hours, formed by the regular revolution of our earth, in which all its inhabitants partake, is particularly distinguished in the physical economy of man ... It is, as it were, the unity of our natural chronology.

C.W. Hufeland.
(The Art of Prolonging Life)

For my parents.

SUMMARY

The concentration of 5-HT in the rat brain displays a marked 24-hour variation which is 180° out of phase with that observed for total brain TRY levels. This investigation was undertaken to study the factors which might prove responsible for this difference. Previous experiments showed 5-HIAA levels were purely a reflection of 5-HT concentrations i.e. the latter were not low in the dark phase because turnover was high.

TRY in the plasma has the unusual property of binding to albumin and it is the 'free' fraction which is functionally important. A comparison was made of 'free' and total TRY levels in the brain as TRY hydroxylase is unsaturated with substrate and fluctuations in the functional TRY pool may be important in controlling 5-HT levels. A 24-hour variation in 'free' TRY was found, again there was a 180° phase difference with the 5-HT rhythm.

The activity of the two synthetic enzymes TRY hydroxylase and 5-HTP decarboxylase were measured by *in vitro* methods (radiometric and fluorimetric, respectively). No significant 24-hour variation in enzyme activity was evident, suggesting no regulation by synthesis rate.

In vivo studies followed in which a behavioural model of induced hyperactivity, thought to reflect central serotonergic activity, showed a 24-hour variation in hyperactivity, the peak level occurring in the latter part of the light period. The effect of re-uptake blockade on this syndrome indicated a reduced rate of re-uptake coinciding with increased hyperactivity rather than an increased rate of 5-HT release.

In the light of these results, the possibility is considered that regulation of 5-HT levels is not via synthesis but that 5-HT is produced in excess and that functional control may be brought about by intraneuronal binding and metabolism.

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CHAPTER ONE

Introduction

INTRODUCTION

5-Hydroxytryptamine (5-HT) is widely distributed in the central nervous system (CNS) of mammals, and there is evidence that it functions there as a neurotransmitter. It has been postulated that serotonergic neurons are involved in numerous functions in the brain, including regulation of various types of behaviour, body temperature, food intake, neuroendocrine function, sleep, and sexual activity.

The concentration of 5-hydroxytryptamine in the rat brain displays a marked circadian variation and it is hoped that the results presented in this thesis will elucidate the biochemical factors which control this persistent rhythm. It is also hoped that through this work a more general picture of the way in which the functional activity of serotonergic neurons are controlled in the brain may emerge.

It is the purpose of this chapter to introduce the reader to the characteristics of circadian rhythms which are particularly relevant to this work and to give a resume of previous investigations involved in the discovery, isolation, distribution and metabolism of this amine and to shed light on its role in the CNS.

1.1 Circadian Rhythms

Life on earth is and always has been exposed to strong and rhythmic environmental changes caused by planetary movements. Thus the rotation of the earth on its polar axis gives rise to the dominant cycle of day and night; the revolution of the earth around the sun gives rise to the procession of the seasons; and the more complicated movements of the moon in relation to the earth and the sun give rise to the lunar month and to the tidal cycles.

During their long evolutionary history, organisms have developed various endogenous rhythms whose periods match those of the environmental changes outlined above, eg. cycles of day and night (circadian, period 24 hours), lunar (circalunar, period 29 days), tide (circatidal, period 12.4 or 24.8 hours), the seasons (circannual, period a year), or the time between successive spring and low waters (semilunar or cirasyzygic, period 14.7 days). The best known of these rhythmic phenomena is the circadian (from cira, about; dies, a day).

The cycle of day and night is important for a number of reasons. Practically all the energy entering the biotic environment is derived from the sun, being incorporated by green plants during the day but not at night. Correlated with this rhythm in light intensity are the rhythms of temperature and humidity; in other words, the three most important environmental variables have a strong 24-hour variation.

Circadian rhythms, however, are those that persist when all environmental fluctuations are excluded,and in this free-running condition show a natural period which is

1) close to but rarely equal to that of solar day

2) very accurate

3) temperature - compensated.

These circadian rhythms may be observed at all levels of organisation except, apparently, in the prokaryotes. Unicellular organisms, particularly algae, show circadian rhythms of phototaxis cell division, photosynthesis and bioluminescence (Sweeney, 1969). Fungi, such as Neurospora spp. and Pilobolus spp. show a 24-hour rhythm of sporulation, although other fungi may show a variety of rhythms with a non-circadian pattern (Jerebzoff, 1965). 'Higher' plants and animals show a vast array of rhythms, some of the most frequently studied being leaf movements of plants, and the sleep-wake or activity cycles of insects, birds and mammals. In whole populations, certain 'one-in-a-lifetime' events, such as egg hatch, moulting, pupation, and adult emergence of insects, show a pronounced circadian rhythm in a mixed-age population. One of the most intensively investigated of such systems is the rhythm of pupal ecolsion in the fruit fly Drosophila pseudoobscura (Pittendrigh, 1954, 1960, 1966). Even cultured cells, tissues and organs may show persistant circadian rhythms when isolated from the whole organism. Thus in plants, isolated sections of leaves continue to show diurnal fluctuations in turgor pressure, growth and CO_2 output (Wilkins, 1960). In animals, isolated sections of the gut of

hamsters show circadian rhythms of peristaltic activity (Bünning, 1973), isolated eyes of the marine mollusc Aplysia californica a rhythm of optic nerve activity (Jacket, 1969) and isolated salivary glands from Drosophila melanogaster a rhythm in nuclear volume (Rensing, 1969).

In a natural 24-hour variation of light and temperature, the circadian system of an animal or plant, which is also oscillatory and with a near-24-hour period of its own becomes entrained to that of the environment in much the same way that two physical oscillators will achieve mutual entrainment. This entraining agent is commonly called a zeitgeber. When exposed to such an environmental zeitgeber the endogenous circadian oscillation adopts the exact 24-hour variation of the driver and also adopts a particular phase relationship to it. By achieving steady-state entrainment to the zeitgeber, an animal or plant can partition its activities with some kind of temporal order, and thereby perform behavioural and physiological activities at the 'right time of day.' Many animals, for example, restrict their locomotor and feeding activities to the hours of darkness (nocturnal), whereas others restrict their activity to the daylight hours (diurnal); still other animals are dusk-active, or crepuscular. The selective advantage of this behaviour may lie in the reduction of direct competition between, for example, diurnal and nocturnal species using the same food source.

There has been much debate as to whether circadian rhythms are

truly endogenous or exogenous. However, when organisms as diverse as unicellular algae, flowering plants, insects, birds and mammals are isolated and placed at constant temperature and in continuous darkness, and with all other possible zeitgebers, such as those caused by periodic human activity in the laboratory, excluded, the observed rhythms still persist, thereby revealing their endogenous nature. In the absence of the zeitgeber however, the rhythm 'free-runs' and the natural period, τ becomes either slightly less or slightly more than 24 hours. The value of τ is generally between 22 and 28 hours, depending on the organism. Thus the rhythm may rapidly become out of phase with the solar day; for an organism whose is 23 hours, for example, the overt phase of the rhythm would occur one hour earlier each day, and would be 180° out of phase with environmental time within two weeks. In animals the range is generally between 23 and 26 hours. The free-running period, however, is very rarely exactly 24 hours, and if it were one would suspect that a natural zeitgeber associated with the solar day had not been excluded. An interesting phenomenon has been observed when animals are kept in continuous light; the circadian rhythms persist but their period τ is altered. The studies led to a generality called 'Aschoff's rule' which states that the free-running period τ in nocturnal animals is longer in continuous light (and is increased still further if light intensity is involved) than in continuous darkness whereas in diurnal animals the situation is reversed. This rule is widely applicable to vertebrate species but finds some exceptions amongst insects.

Pittendrigh's work (1954) on eclosion rhythm of Drosophila pseudoobscura focused attention on the phenomenon which is an essential functional prerequisite for a biological clock. He showed that the endogenous rhythm was temperature compensated. An oscillator which 'ran faster' as the temperature rose would be useless as a 'clock' and in the absence of compensation the period of the biological oscillator would at most temperatures fall outside the range of entrainment by the environmental zeitgeber and thus another important aspect i.e. control of phase would cease to function. Most physiological processes double their rate with a 10° rise in temperature yet the period of a circadian oscillator in its free-running state retains approximately the same value within a wide range of ecologically important temperatures. This effect seems strange when considering birds and mammals which have closely regulated 'core' temperatures and in which temperature compensation as such, might not be expected. A number of species of mammals have been subjected to deep hypothermia for varying periods and the effect of this treatment on their rhythms examined eg. in mice and hamsters, hypothermia down to 5° for 3-8 hours had little delaying effect on the activity rhythm (Rawson, 1960). This work suggests that this feature, important for chronometric purposes, is older in an evolutionary sense than the mammalian homeostatic temperature regulation.

The ability of organisms to adapt to external variations particularly solar or lunar rhythms has led to the idea that they possess some sort of timing mechanism. In particular,

the idea of an endogenous 'biological clock' which is responsive to and which can be synchronised, reset and otherwise modulated by environmental variations, such as that mentioned above, which the organism has encountered and to which it has adapted, throughout its evolutionary history. The nature of this 'clock(s)' remains an enigma. Some workers suggest that the nucleus and protein synthesis are closely connected with the ciradian 'clock', whilst others believe that membrane physiology, cytoplasmic organelles, transcription along the DNA tape or high frequency biochemical oscillations are involved (see Edmunds, 1976 for review). Since true circadian rhythms have not been recorded from isolated sub-cellular particles or from cell-free extracts, it is probable that the 'biological clock' does not lie within the cell, but is the cell, the spatial integrity of the cell and interactions between organelles being essential for the special features of circadian variation. In a theoretical study, Winfree (1967) demonstrated how a population of oscillators, all with a similar fluctuation and a weak mutual coupling, tend to synchronise each other to a common phase and period, even though starting at random phase to each other. Perhaps it is in this way i.e. entrainment to a 24 hour environmental zeitgeber that all the constituent cellular oscillators achieve a mutual phase-relationship in multicellular organisms to provide whole-organism synchrony. This may explain why 'free-running' can fade out after a few days or weeks; in the absence of the zeitgeber, the various endogenous components may desynchronise.

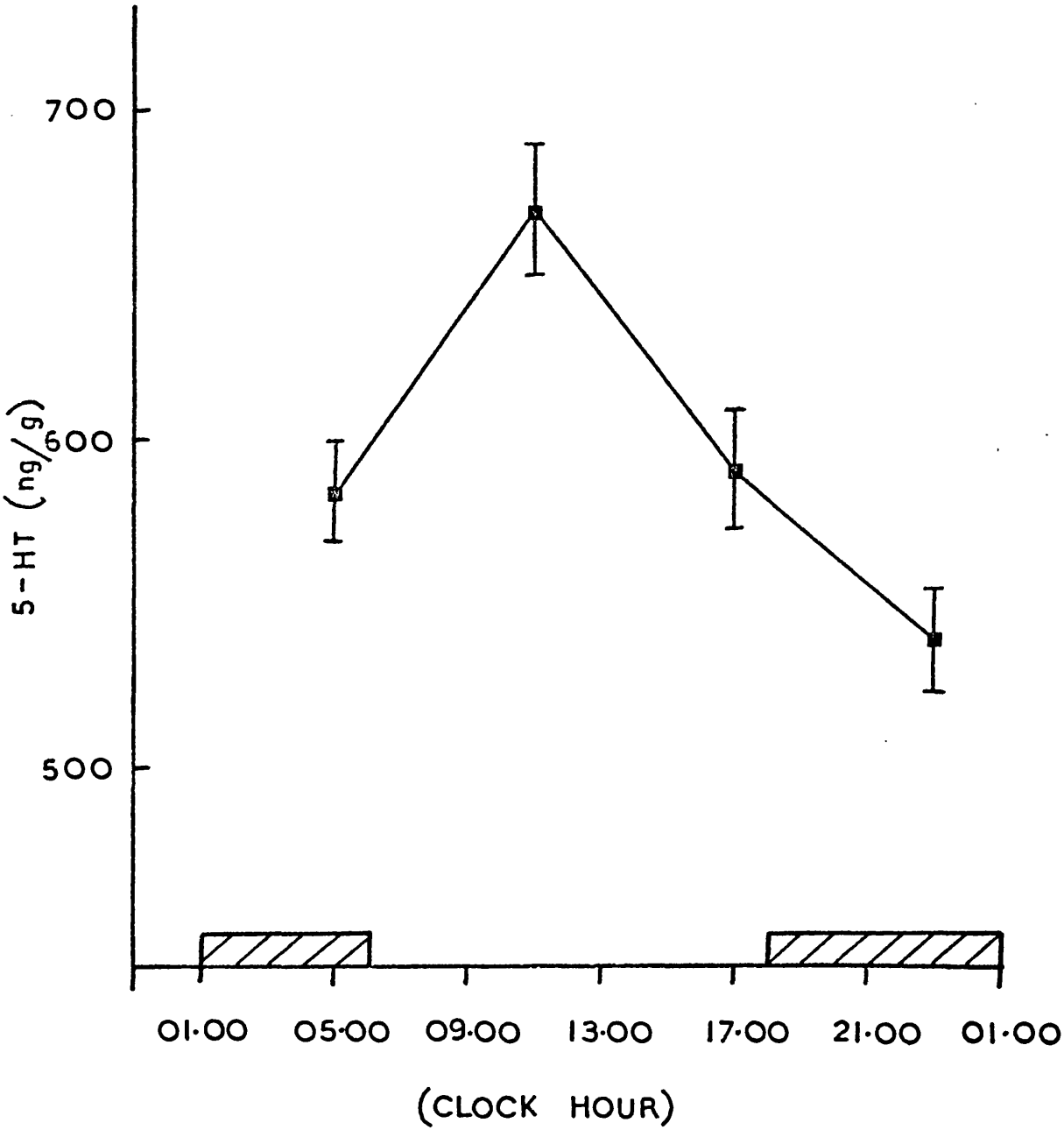


Fig.1 Circadian rhythm of endogenous 5-HT levels in the rat brain

The work presented here has not attempted to locate a 'biological clock' but has investigated factors involved in the metabolism of 5-HT which could be capable of regulating the circadian rhythm of this amine in the rat brain. This influence itself may subsequently be controlled by a 'clock'. This may also provide more evidence about the way in which the functional activity of this neurotransmitter is controlled in the brain.

1.2 Evidence for a circadian rhythm of 5-HT in brain

A circadian rhythm in the concentration of 5-HT levels has been demonstrated in the whole brain of the mouse (Albrecht et al., 1956; Morgan and Undo, 1973), in the whole brain of the rat (Dixit and Buckley, 1967; Scheving et al., 1968; Schwartz and Aghajanian, 1969; Okada, 1971; Asano, 1971 and Davies and Redfern, 1970), and the whole brain of the hamster (Matussek and Patshke, 1963).

In whole rat brain, the highest concentration of 5-HT was found during the light period, and the lowest concentration during the dark period. This has also been confirmed in the work presented here (Fig. 1). A similar situation was also seen in brain regions of the rat e.g. the hypothalamus (Quay, 1968; Hery et al., 1972), the cortex (Quay, 1965; Hery et al., 1972), the whole brain stem (Hery et al., 1972), the lateral lower brain stem (Quay, 1968), the mid-brain and caudate nucleus (Friedman and Walker, 1968) and the amygdala and hippocampus (Scapagnini et al., 1972). In all these instances

the highest 5-HT levels were in the light period thus it would appear that the summation of the circadian effects on the concentration of 5-HT in the brain regions is responsible for the large fluctuation measured in whole brain. As whole brain measurements thus reflected levels in brain regions, the former was used as the tissue of study in this thesis.

1.3 Historical Introduction

Since the middle of the nineteenth century, scientists have been aware of a vasoconstrictor substance found in the serum of clotted blood which had the ability to cause powerful constriction of smooth muscle organs. Not until the mid-twentieth century did scientists at the Cleveland Clinic in America succeed in isolating this substance as a possible cause of high blood pressure and gave it the name 'serotonin' (Rapport *et al.*, 1948). The following year Rapport (1949), characterised the active moiety as 5-HT. When this compound was prepared synthetically by Hamlin and Fischer in 1951, it was found to behave exactly as the naturally occurring 'serotonin?'

At the same time, Erspamer and his co-workers in Italy were trying to characterise the substance found in high concentrations in enterochromaffin cells of the intestinal mucosa. This material had the ability to cause constriction of smooth muscle, particularly the gut. The material isolated from the intestinal tract was called 'enteramine'. By 1946 Erspamer had proposed that the active component was an indolealkylamine

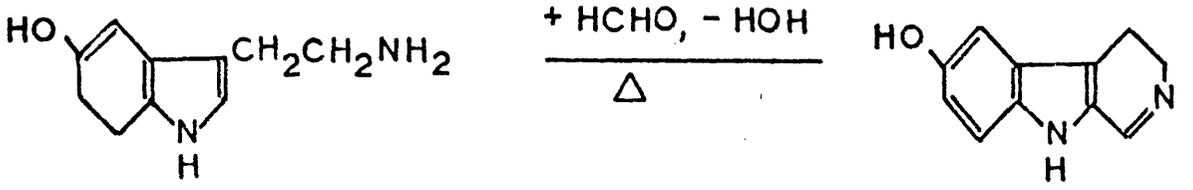
but he was unable to characterise it further until 5-HT had been discovered in the blood. Erspamer and Asero (1952) then established that this was the chemical nature of 'enteramine'.

1.4 The Occurrence of 5-HT

Indolealkylamines, including 5-HT, occur widely in nature for example, many types of fruit such as pineapples and bananas contain extremely large amounts of 5-HT. Also it is present in stings and verom e.g. common stinging nettles, wasps and scorpions. Mammals, birds, reptiles, amphibians, fish, tunicates, molluscs, arthropods and collentrates have all been shown to contain 5-HT. In mammals the highest concentration of this amine occurs in the pineal gland and in the enterochromaffin cells of the intestinal tract. In man, it is estimated that about 90% of the body's 5-HT occurs in the gastrointestinal tract, another 8 to 10% in platelets and only 2-5% within the central nervous system.

1.5 5-HT in the CNS

The studies of Amin et al., (1954) were the first to show the existence of appreciable amounts of 5-HT in the brains of dog and also its uneven distribution. However, it was nearly ten years before scientists developed techniques to show that 5-HT in the brain was contained in neurons. The first support for this view arose from sub-cellular fractionation studies showing 5-HT and 5-hydroxytryptophan decarboxylase to be associated with pinched-off nerve endings or synaptosomes (Zieher and De Robertis, 1963).



3, 4 - dihydro - β - carboline

Apparent colour 'Yellow'

Fluorescence

Excitation / Emission 425, 470 / 530 nm

FIG. 2 Formaldehyde condensation product of 5-HT

While this finding suggested only a general relationship to nerve terminals, two other techniques evolved which related 5-HT to specific nerve tracts more closely. One technique involved combining neuro-anatomical lesioning with biochemical measurements and it was shown that lesions placed in the median forebrain bundle of the hypothalamus led to a dramatic fall in forebrain 5-HT concentrations over the next 5 to 12 days (Heller and Moore, 1968).

The second technique of fluorescent histochemistry has perhaps achieved most in associating the amine with specific neurons. Barker and Pearse (Pearse, 1960) originally developed a fluorescent B-carboline complex (Fig. 2) from the addition of one molecule of formaldehyde to the 5-HT molecule. The method was adapted for the mapping of neurons containing 5-HT in the brain by the Swedish group of workers (for summary see Fuxe, Hokfelt and Ungerstedt, 1968). Unfortunately, the fluorescence technique for 5-HT is not as sensitive as that for noradrenaline (NA) and dopamine (DA) but various modifications of 5-HT concentrations have been applied to emphasize 5-HT-containing neurons (Dahlström et al., 1973), for instance, Aghajanian and Asher (1971) used tryptophan (TRY) loading to increase 5-HT concentrations in neurons and consequently increase fluorescence. Also the fluorescent product developed after incubation of brain slices with 6-hydroxytryptamine has a greater intensity. Unfortunately, 6-hydroxytryptamine is non-specific i.e. it can be taken up by catecholaminergic neurons also. This can be overcome if

the latter are selectively destroyed first by pre-incubation with 6-hydroxydopamine (Farnebo 1971).

All these studies indeed provide evidence that 5-HT is contained within specific neurons in the brain. The ultrastructure of the nerve terminal is similar to that of catecholaminergic neurons. Both large and small dense core vesicles are present at least in the hypothalamus (Aghajanian, Bloom and Sheard, 1969). Also the amine shorage granules appear to be of more than one type (Thierry, Fekete and Glowinski, 1968; Shield and Eccleston, 1972), and perhaps represent two forms, one important for release and the other for storage.

1.6 The Distribution of 5-HT in the CNS

The histochemical fluorescence technique and quantitative biochemical analysis mentioned previously have been used together with CNS lesioning to give much information on the mapping of 5-HT pathways. The principle of neurochemical lesioning was extended from the catecholamines, using 6-hydroxydopamine (Tranzer and Theoren, 1967) to serotonergic neurons by the use of 5,6-dihydroxytryptamine (Baumgarten and Lachenmayer, 1972a; Nobin et al., 1973) and 5,7-dihydroxy-tryptamine (Baumgarten and Lachenmayer, 1972b). These drugs cause acute degenerative changes in terminal ramifications and reactive changes in the preterminal portions of serotonergic neurons following their uptake. Preliminary data suggest that 5,7-dihydroxytryptamine is better tolerated in higher doses and that these doses cause greater damage than a

5-HT PATHWAYS

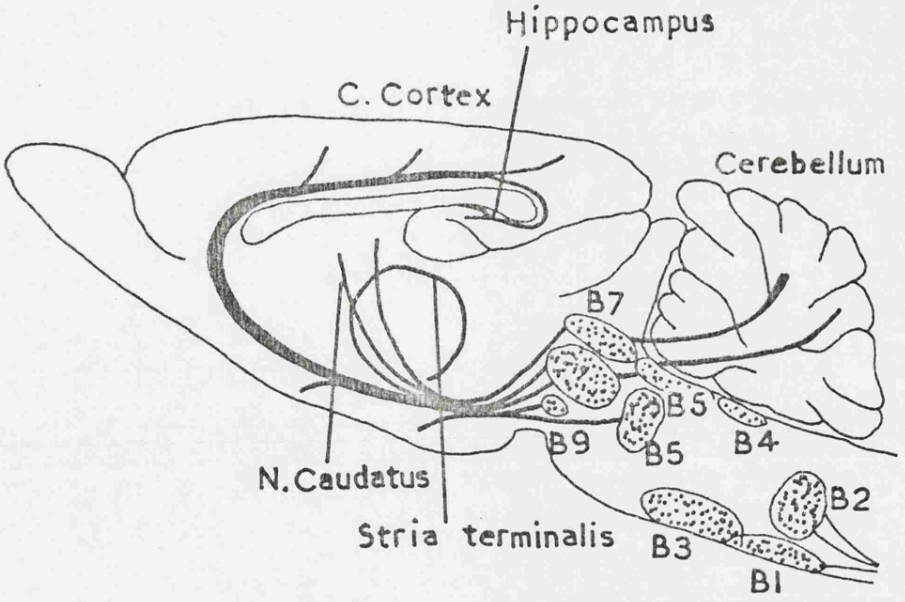


FIG. 3a. Schematic diagram of the central 5-HT neurons (modified from Fuxe, Hökfelt, Olson, & Ungerstedt, 1973).

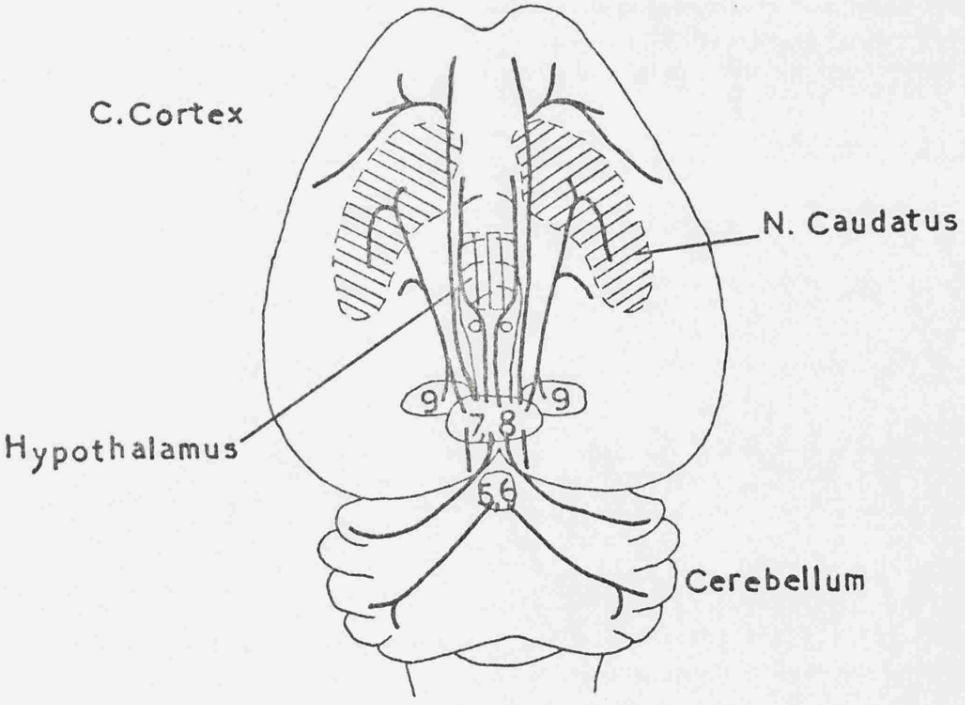


FIG. 3b. Schematic illustration of subdivision of the ascending 5-HT pathways

tolerated dose of 5,6-dihydroxytryptamine (Baumgarten et al., 1973b). 5-HT contained in the vesicles that accumulates proximal to the lesion may be better visualised while distal to the lesion, the nerve terminals and axons degenerate and the amine content falls.

These methods have shown that most of the cell bodies occur in the mid-brain (B7, B8, B9),pons (B5, B6) and medulla (B1, B2, B3). The nucleus raphe dorsalis (B7) is the largest 5-HT-containing raphe nucleus (Dahlström and Fuxe, 1965; Hökfelt et al., 1973). These nuclei give rise to an ascending system of axons which begin to coalesce into a compact bundle at the level of the interpeduncular nucleus (Ungerstedt, 1971). The axons then occupy the most ventral part in the median forebrain bundle and may be separated into a medial and lateral component (Fig. 3). The lateral ascending 5-HT pathway primarily innervates the cortical areas and a minor, far-lateral pathway may exist which primarily innervates the extrapyramidal motor system. These pathways originate mainly from the mesencephalic raphe cell group i.e. B7, B8 and B9. The medial ascending pathway is subcortical and innervates the hypothalamus and the pre-optic area. This pathway originates from the mesencephalic raphe (B7 and B8) and also the pontic raphe region (B5 and B6). The cerebellum innervation originates mainly from the mescencephalic raphe (B7 and B8) and possibly raphe cell groups (B5, B6) in the pons (Bloom et al., 1972; Fuxe and Jonsson, 1974).

The most caudal 5-HT cell groups in the medulla oblongata (B1 - B3) give rise to axons that descend in the medial part of the anterior funiculus and in the anterior part of the lateral funiculus to innervate the ventral horn and other axons that descend in the dorsal part of the lateral funiculus to innervate the dorsal horn and the symapathetic lateral column. (Carlsson et al., 1964; Dahlström and Fuxe, 1965; Ungerstadt, 1971).

As stated previously, it is difficult to visualise 5-HT nerve terminals because of the insensitivity of the Falck-Hillarp technique. They have been mainly demonstrated in the nucleus suprachiasmaticus, globus pallidus, certain amygdaloid nuclei and in the ventral part of the lateral geniculate body (Fuxe et al., 1968). After treatment to increase the fluorescent intensity (discussed in section 1.5), very fine 5-HT nerve terminals were observed in the septal area, in the anterior colliculi of the preoptic area, in the hippocampal formation and in the neo-and mesocortex. (Dahlström and Fuxe, 1964). With the use of 6-hydroxytryptamine (see section 1.5) it was possible to visualise a scanty 5-HT nerve terminal system in the nucleus caudatus putamen (Fuxe and Jonsson, 1974).

Biochemical microtechniques provide a significant advantage in precise quantitation of the amount of 5-HT present as estimates based on the intensity of the fluorescence observed under the microscope sometimes proved to be inaccurate.

However, the former method gives no information about the nature of the structures containing the 5-HT i.e. neuronal cell bodies, terminals, axons or glial cells. Using biochemical methods, the areas found to contain the greatest amounts of 5-HT do agree with results from histofluorescence techniques. In the hypothalamus and preoptic area, high levels were detected in the nucleus suprachiasmaticus and the nucleus preopticus suprachiasmaticus. The limbic system was relatively rich in 5-HT particularly the amygdaloid nuclei. There was an eight-fold difference in this area between the lowest concentration in the hippocampus and the highest in the posterior medial forebrain bundle. In the rostral limbic system the highest 5-HT levels were found in the tuberculum olfactorium, the nucleus interstitialis striae medullaris and the nucleus interstitialis striae terminalis. In the brain stem high concentrations of 5-HT were found in the raphe nuclei and regions known to contain large numbers of serotonergic nerve terminals (e.g. motor cranial nerve nuclei, substantia nigra, globus pallidus). All three parts of the substantia nigra were strikingly rich in 5-HT compared with structures near them. 5-HT seemed to be evenly distributed between the various components of the reticular formation, amounts being moderate. Levels in the cerebellar cortex and nuclei were low (Saavedra, 1977).

1.7 Evidence for release and role of 5-HT synaptic transmission

The localisation of synthesising enzymes and of 5-HT itself and the organisation of the nerve ending in this regard do suggest that 5-HT might be a neurotransmitter in the classical

sense. The criteria established by Chang and Gaddum (1933) for the role of acetylcholine as a neurotransmitter agent are here applied to 5-HT.

1. It should exert an action which elucidates its physiological role. In the CNS, this is a difficult criterion to meet. Certainly, when applied by microelectrophoretic techniques to selected neurons, 5-HT exerts effects (Bloom et al., 1972), but whether those reflect its physiological effects is unknown.

2. It should be manufactured in the neuron. That criterion is met.

3. It should be destroyed by an enzyme present in nervous tissue. That criterian is met, and in addition it is now known that inactivation by reuptake into presynaptic nerve endings occurs (Snyder et al., 1973).

4. It should be potentiated in vivo by factors which inhibit its enzymatic degradation in vitro; that criterion is met by the effects of monoamine oxidase (MAO) inhibitors.

5. It should be antagonised in vivo by specific chemical inhibitors. Certainly microelectrophoretic studies have shown that both lysergic acid diethylamide (LSD) and methysergide can block 5-HT responses by certain neurons, but both of these agents have pharmacological 'depressant' actions in their own rights (Bloom et al., 1973).

The question to be asked is, "Does stimulation of a 5-HT neuron cause a release of 5-HT which then has a pharmacological effect (either excitatory or inhibitory) on the post-synaptic membrane?" At present this cannot be answered as there is no

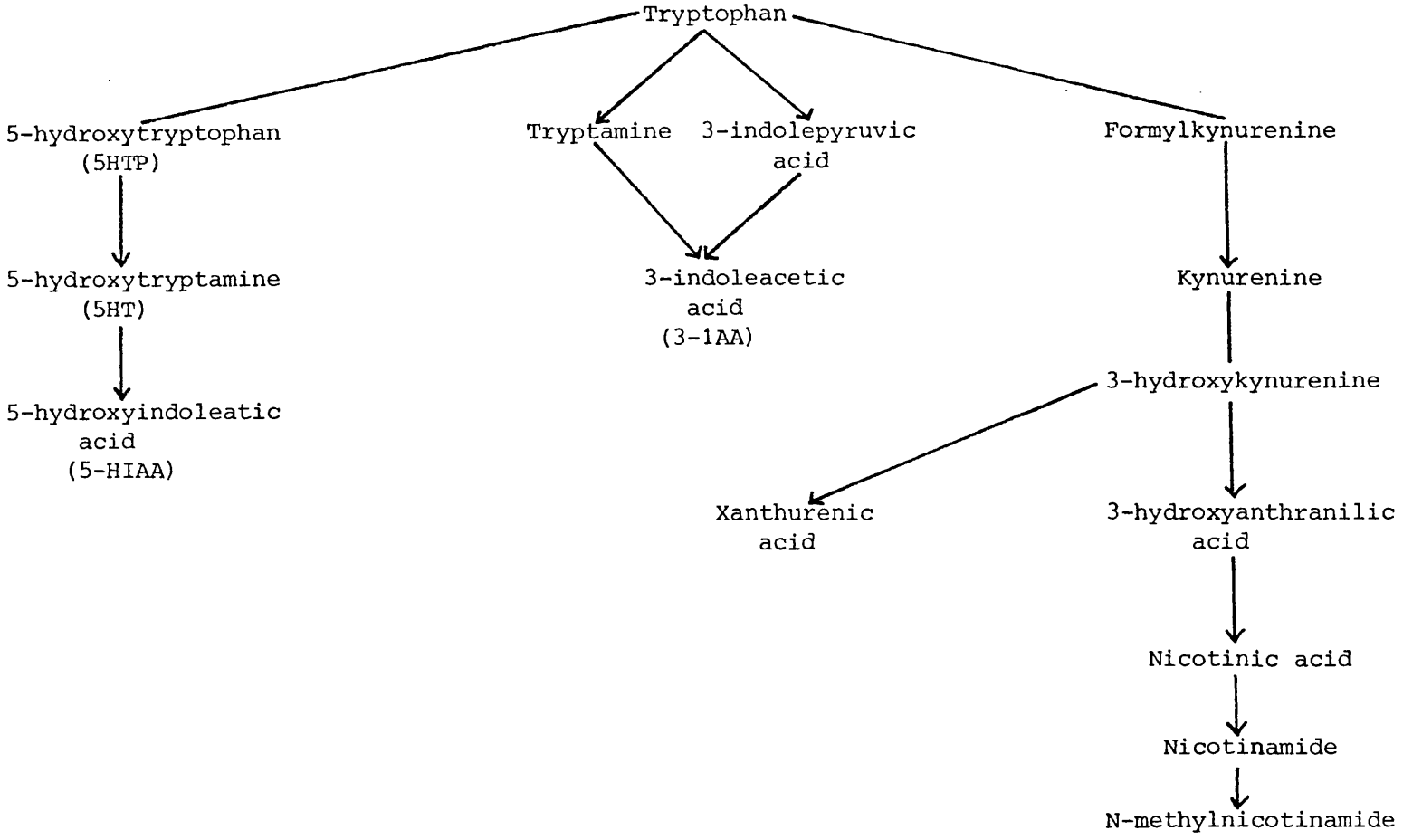


Fig. 4 Pathways of tryptophan metabolism

direct evidence for such an effect.

In molluscs, 5-HT does appear to subserve a neurotransmitter role (Gershchenfield and Stefani, 1968) so a precedent is available. Electrical stimulation of brain slices undoubtedly causes the release of 5-HT (Chase et al., 1969). Also, perfusion of the lateral or third ventricle of a cat's brain with artificial cerebrospinal fluid (CSF) showed the appearance of 5-HT in the perfusate when the two most rostral raphe nuclei were stimulated electrically (Holman and Vogt, 1972; Ashkenazi et al., 1973). However, it cannot be certain that this has direct bearing on the question.

However, the experiments described in this thesis were designed on the assumption that 5-HT is a classical neurotransmitter; should this turn out not to be the case, many theories and speculations on the role of 5-HT in the brain, on the organisation of its synthesis and metabolism, and on the mode of actions of drugs will need total re-evaluation.

1.8 The Metabolism of 5-HT

Peripherally, the major route of metabolism of the amino acid precursor of 5-HT, TRY, is its oxidation to formylkynurenine by liver pyrrolase to form formylkynurenine and by formamidation to kynurenine - the principal metabolite (Fig. 4). However, there is no evidence to suggest the presence of pyrrolase in the CNS and none to suggest the

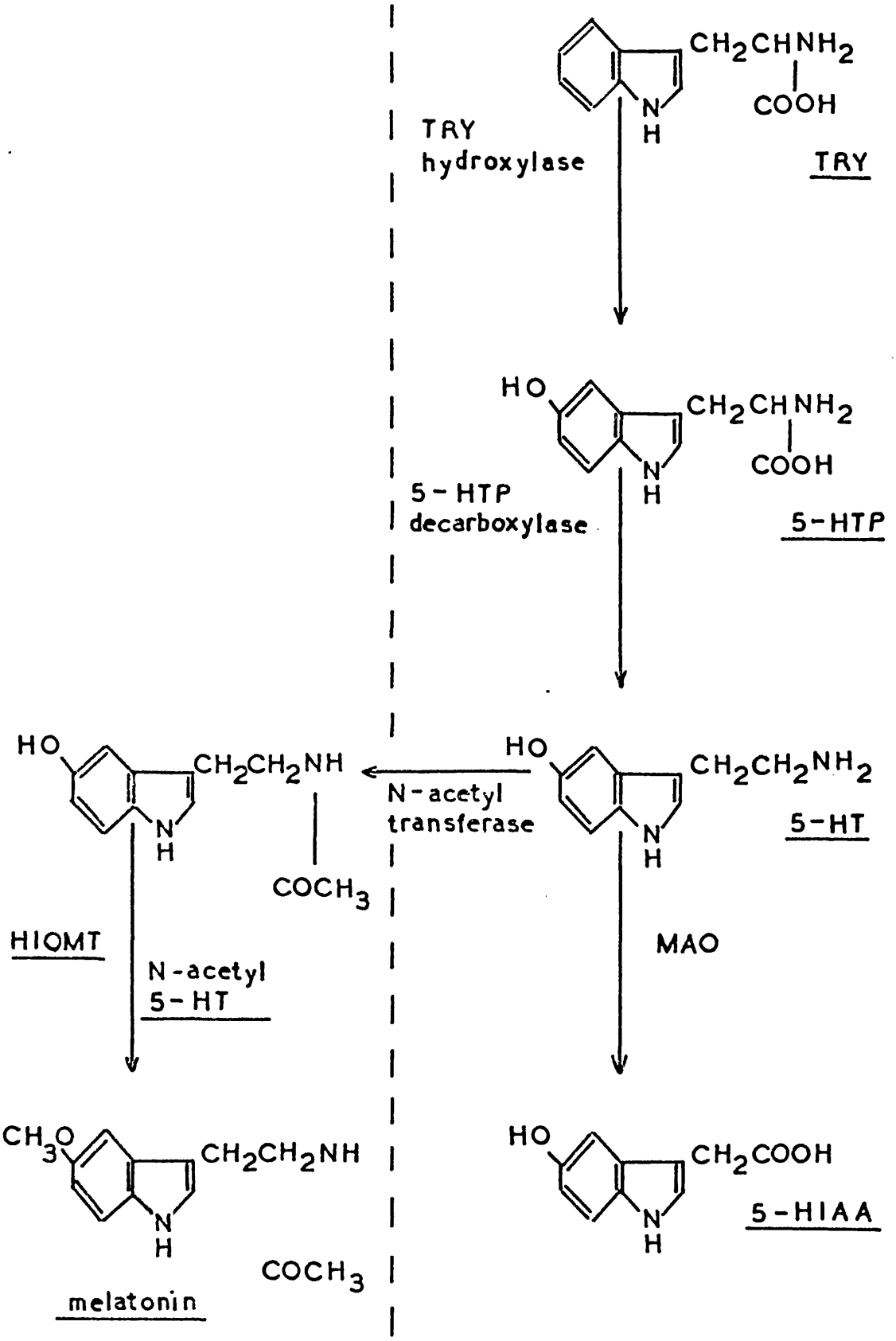


FIG. 5

The metabolism of 5-HT in the pineal gland

The major route of metabolism of 5-HT in the rat brain

existence of kynurenine. Also TRY can be decarboxylated in the periphery to form tryptamine.

In the CNS, it has been established that 5-HT is synthesised by hydroxylation to 5-hydroxytryptophan (5-HTP) and decarboxylation to the amine. It is catabolised by MAO to the major excretory product 5-hydroxyindoleacetic acid (5-HIAA) (Udenfriend et al., 1956) (Fig. 5). The distribution of the enzymes necessary for 5-HT synthesis and breakdown closely follow that for 5-HT and are located in, or associated with nerve endings, axons and cell bodies which have been shown to contain the amine. This pathway is the major metabolic pathway of 5-HT and the investigation of the components of this pathway form a major part of the work presented in this thesis and are therefore discussed in greater detail in the following chapters.

However, alternative routes of metabolism do exist in the CNS and these are considered in the next section.

1.9 Other indoleamines in the CNS

1.9.1 Melatonin, 5-Methoxytryptamine and N-methylation

Any review of indoles other than 5-HT in the CNS has to include the indoleamine pathways in the pineal gland. This is something of a contradiction since the pineal is

cytologically outside the brain and its innervation comes not from the brain but from the superior cervical ganglion. Its inclusion is necessary since anatomically it is closely associated with the brain and it appears to have close pharmacological connections, synthesising compounds which have been shown to alter brain function.

That mammalian pineal extracts will lighten amphibian skin has been known for many years. However, the active substance was not isolated until 1959, and was identified as 5-methoxy-N-acetyltryptamine or melatonin (Lerner et al.,1959).

The synthesis of melatonin is represented in Fig. 5. It appears to be controlled by the light-dark cycle. The activity of 5-HT-N-acetyltransferase increases in rats in the dark to 15-70 times the daytime values (Klein and Weller, 1970; Ellison et al., 1972). Exposure of rats to light results in a drop in activity of this enzyme, with activity halving in about 3 min. Physiologically it seems reasonable to relate this alteration in enzyme activity to control of pineal melatonin production and findings with pineal glands in organ culture suggests that both the production and the secretion of melatonin are controlled in this way (Klein and Weller, 1970; Klein et al., 1970,1971; Ellison et al., 1972). It is thought that the visual signals of the light-dark cycle regulate the release of melatonin and are thus converted to an endocrine signal (Wurtman and Anton-Tay,1969) that may have special importance in seasonal breeders such

as the ferret, which becomes sexually active when the dark period is shortened (Herbert, 1971). In other animals, it appears to have some fairly direct effects on the reproductive cycle. Implants of melatonin (Fostini et al., 1968) or perfusion of melatonin into the third ventricle (Kamberi et al., 1970) results in a stimulation of prolactin release and an inhibition in release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Several differences are evident in the hydroxylase enzyme in this system when compared to that of the brain. Hydroxylase activity in the pineal gland is unusually high (Lovenberg et al., 1967) and work with p-chorophenylalanine (PCPA) has suggested that this compound does not inhibit the pineal enzyme directly, as in the brain, but competes for uptake with the substrate TRY (Deguchi and Barchas, 1972). However, the enzyme is stimulated in pineal gland culture by addition of mescaline (Shein et al., 1971) which is consistent with the effects of this compound on whole brain. Another difference in 5-HT formation in the pineal gland is that constant lighting will increase decarboxylase activity (Snyder et al., 1965) although the physiological importance of this is not clear (Axelrod and Wurtman, 1968). Finally, the 5-HT in the pineal is localised differently from that in the brain, being present in both parenchymal cells and sympathetic nerve endings (Bertler et al., 1964), with 5-HT in these two compartments having different turnover rates (Neff et al., 1969b).

The fact that hydroxyindole O-methyltransferase (HIOMT) appeared to be unique to the pineal in mammals (Axelrod and Weissbach, 1961) precluded the formation of melatonin elsewhere. However, pineal HIOMT uses S-adenosylmethionine (SAM) as its methyl donor (Axelrod and Weissbach, 1961) and these workers used SAM as the donor when searching for HIOMT activity in the brain. Laduron (1972) has shown that 5-methyltetrahydrofolic acid is the probable methyl donor in the conversion of DA to epinine in the adrenal medulla and this prompted Banerjee and Snyder (1973) to investigate the possibility that this compound is also a methyl donor for 5-HT in the CNS. They obtained significant O-methylating activity. The product being 5-methoxytryptamine (5-MT). This is consistent with the observations of Green et al., (1973) who had already reported the presence of significant amounts of 5-MT in the hypothalamus using gas chromatography-mass spectrometry.

Having shown evidence of O-methylation of 5-HT in the brain, there was considerable interest in the possibility of N-methylation of indoleamines, since some synthetic N-methylated indoles have been shown to be potent psychotomimetic agents, and abnormal N-methylation has been suggested to occur in certain mental illnesses (Szara, 1956; Pollin et al., 1961; Tanimukai et al., 1970; Mandell and Spooner, 1968). An indoleamine N-methyltransferase was shown to occur in chick and human brains (Morgan and Mandell, 1969; Mandell and Morgan, 1971) and other mammalian tissues (Saavedra and

Axelrod, 1972b). These experiments used the universally accepted methyl donor SAM, and enzyme activity was low. Banerjee and Snyder (1973) using 5-methyltetrahydrofolate while finding significant O-methylating activity as stated previously, found little evidence of N-methylation of 5-HT. Saavedra and Axelrod (1972b) however, after showing tryptamine to be present in the brain, demonstrated its conversion in rat and human brain to N-methyl and N,N-dimethyltryptamine. Banerjee and Snyder (1973) confirmed this and also demonstrated O-methylation of 5-hydroxy-N-,N-dimethyltryptamine (bufotenin) to 5-methoxy-N,N-dimethyltryptamine (5-MeODMT) a potent psychedelic agent (Holmstedt and Lindgren, 1967). Bufotenin has been reported to occur in the plasma and urine of schizophrenics (Narasimhachari et al., 1971) together with 5-MeODMT and N,N-dimethyltryptamine. However, since normal plasma also contains an N-methylating enzyme (Wyatt et al., 1973b) these compounds need not have originated in the CNS. If these compounds do occur in the CNS in certain pathological conditions, it is possible that their formation is suppressed in the normal state, and there is some evidence for this. 5-HT, catecholamines and histamine may inhibit tryptamine methylation since they are substrates for the nonspecific methylating enzyme (Axelrod, 1961,1962) which has a specific distribution pattern in the brain (Saavedra et al., 1973) and S-adenosylhomocysteine, which occurs normally in brain, is reported to inhibit several methyltransferases (Deguchi and Barchas, 1971) and could thus in part control N-methylation in the brain.

1.9.2 Tryptamine

Decarboxylation of TRY would produce tryptamine. The presence of high 5-HTP decarboxylase activity in the brain suggests that this compound could be present.

Björklund and Falck (1969) identified a tryptamine-like compound in the pituitary using histochemical techniques, and this was followed by the report of Martin et al., (1972) on the presence of tryptamine in normal steer, dog and human brain using gas chromatography and spectrophotofluorimetry. These workers were unable to find the compound in the normal rat brain. However, after development of a sensitive and specific enzymatic-radiochemical method (Saavedra and Axelrod, 1972a), tryptamine was demonstrated in normal rat brain (Saavedra and Axelrod, 1972b).

Iproniazid or TRY was shown to cause a small but significant increase in tryptamine, with a larger increase when both compounds were injected. PCPA also increased brain tryptamine, with a larger increase when TRY was given with it. These two drugs plus iproniazid increased tryptamine still further. Reserpine was without effect but a central decarboxylase inhibitor, NSD 1055, decreased levels by 40% and blocked the rise associated with iproniazid and TRY (Saavedra and Axelrod, 1973).

Similar results on the effects of MAO inhibitors, PCPA and reserpine were obtained by Marsden and Curzon (1974). Snodgrass

and Horn (1973) showed that the spinal cord is richer than the brain in this amine and that concentrations increase rapidly after death. The brain tryptamine concentrations were highest in the hypothalamus and striatum and lowest in cerebral cortex and cerebellum.

Marsden and Curzon (1974) commented that the absence of any parallel distribution of 5-HT and tryptamine argues against tryptamine being synthesised specifically in serotonergic neurons. This view was supported by their work using lesions and 6-hydroxydopamine which indicated that TRY was being decarboxylated to tryptamine in both serotonergic and catecholaminergic neurons. They found no synaptosomal uptake mechanism for {^{3}H}tryptamine, which is consistent with the observation that intracerebrally injected tryptamine disappears rapidly from brain (Meek et al., 1970b) and that reserpine has no effect on brain tryptamine concentrations (Saavedra and Axelrod, 1973; Marsden and Curzon, 1974). The conclusions drawn from this work are that tryptamine is not formed in specific neurons and cannot be stored intraneuronally.

1.10 The Role of 5-HT in Behaviour and Physiological Systems

1.10.1 Homeostasis; autonomic functions.

a) Sleep

The first phenomenon shown to depend on the integrity of serotonergic neurons was sleep. Jouvet (1962) discovered that cats given PCPA exhibited a decrease in both slow-wave and paradoxical sleep followed by complete insomnia. The effect could be reversed by the administration of 5-HTP. The action of PCPA could be mimicked by destroying the raphe nuclei of the midbrain and pons.

If indeed 5-HT systems are involved in the production of sleep, the situation is not a simple one. Evidence suggests 5-HT containing pathways interact with catecholamine pathways in the regulation of the sleep-waking cycle. Jouvet (1972) believes that NA rather than DA is involved in waking and paradoxical sleep. After raphe lesions when insomnia has developed and is almost permanent, α-methyl-p-tyrosine produces cessation of running movements, miosis and increased EEG cortical synchronisation, all of which last for 24 hours, after which there is a return to behavioural and ECG insomnia. Lesions of specific NA pathways produce hypersomnia during both slow-wave and paradoxical sleep, and this is accompanied by an increase in brain and spinal cord 5-HIAA, possibly suggesting increased 5-HT turnover.

b) Temperature Regulation

In 1964, Feldberg and Myers suggested that cerebral 5-HT appeared to be involved in temperature regulation. They found that injection of 5-HT into the hypothalamus raised the temperature in the cat. It was then shown that 5-HT was released from the hypothalamus when an animal was cooled (Myers et al., 1969) They implanted a 'push-pull cannula' into the hypothalamus of a monkey and tested the effluent for 5-HT. The animal was subjected to a blast of cold air. The 5-HT content of the perfusate rose by a factor ranging from 2 to 24. Myers and Waller (1975) microinjected 5-HT into the anterior hypothalamus and produced a release of acetylcholine (ACh) into the caudal hypothalamus. This strongly suggested that 5-HT release in the anterior hypothalamic preoptic area is involved in raising body temperature, possibly by triggering the activity of a cholinergic pathway which runs caudally in the diencephalon and mesencephalon. It was also observed that fever produced in a cat by bacterial pyrogen, or by intracerebro-ventricular injection of prostaglandin E, is much reduced after the administration of PCPA (Harvey and Milton 1974). From the evidence presented here, it appears that in the absence of serotonergic neurons an animal has difficulty in raising its body temperature. However, there are puzzling species differences in the role of 5-HT in temperature control. Bligh et al., (1971) found that injection of 5-HT into the lateral ventricle of sheep, goats and rabbits activated mechanisms of heat loss, not of heat production or preservation.

In view of the role of 5-HT containing neurons in temperature and sleep regulation it might be expected that they also play a role in hibernation. Inhibition of 5-HT synthesis in the ground squirrel by PCPA prevents hibernation and raphe lesions inhibit it either partially or completely. (Spafford et al., 1971). There has been no analysis of the multiple mechanisms involved.

c) Respiration

Many workers have experienced the difficulty of respiratory depression when anaethetizing a cat treated with an MAO inhibitor. The phenomenon appears to be due to the accumulation of 5-HT. Since it can also be produced by 5-HTP and since the latter is active in the presence of an inhibitor of 5-HTP decarboxylase injected cerebroventricularly (Armijo and Florez, 1974). This effect may be an example, of the prevention by serotonergic neurons of excessive responses to sensory stimuli, carbon dioxide being the stimulus active in controlling respiration.

d) Vasomotor reflexes

Stimulation of the nucleus raphe obscurus caused a fall in blood pressure and a reduction of spontaneous and evoked sympathetic activity recorded from the white rami communicates; this reduction was mimicked by an intravenous injection of 5-HTP (Neumayr et al., 1974). The pathway involved has its terminals on cells of the intermedio-lateral columns.

1.10.2 Release of pituitary hormones

There is evidence for an inhibitory effect of 5-HT and therefore probably serotonergic neurons, on the liberation of a number of hypothalamic releasing or release-inhibiting factors. The demonstration of 5-HT and serotonergic terminals in the median eminence (Saavedra et al., 1974; Calas et al., 1974) shows a possible morphological basis for such a function. As a result of administering 5-HT, the appearance in the blood stream of pituitary hormones is either increased or decreased, depending on whether their secretion is mainly controlled by a hypothalamic polypeptide which stimulates, or by one which inhibits secretion. Thus it has been shown that secretion of LH and FSH are suppressed while melanophore-stimulating hormone (MSH) and prolactin are enhanced by injection of 5-HT into the third ventricle or by administration of 5-HTP; the opposite effects follow treatment with PCPA. (Kamberi et al., 1970; Kamberi et al., 1971; Kordan et al., 1973/74; Scheider and McCann, 1970; Taleisnik et al., 1973/74; Chen and Meites, 1975).

The effect i.e. inhibition of 5-HT on the liberation of LH releasing factor was visualised by a fluorescent antibody reaction in the guinea-pig brain (Leonardelli et al., 1974). After intraventricular injection of 5-HT, sections of the preoptic and suprachiasmatic nuclei of the hypothalamus reacted with an antiserum to the releasing factor, thus producing fluorescent cells absent from the controls. The interpretation is that, when secretion of the releasing

factor stops, it accumulates in the cells which manufacture it.

An inhibitory effect of 5-HT and 5-HTP has been observed on oxytocin release in the suckling rat (Mizuno et al., 1967). The exact site of action is not yet known. It is interesting that secretion of oxytocin and of prolactin are influenced by 5-HT in opposite directions.

However, the effects of 5-HT on the liberation of hypothalamic releasing or release-inhibiting factors again appears not to be a simple role. Taleisnik et al., (1973/74) showed that not only the injection of 5-HT into the third ventricle but also that of γ-aminobutyric acid (GABA) caused MSH release. Both effects were inhibited by picrotoxin, a known GABA antagonist. The authors suggested a serotonergic neuron is in series with a GABA neuron which in turn inhibits, either directly or by presynaptic inhibition of an tonic stimulatory (adrenergic) system, the neurons producing MSH releasing inhibitory factor.

1.10.3 Motor Activity

Monosynaptic reflexes as well as spontaneous motorneuron activity are exaggerated and polysynaptic reflexes reduced by exogenous 5-HT (Anden et al., 1964; Anderson and Shibya, 1966; Barasi and Roberts, 1974), suggesting that the serotonergic axons in the cord contribute to the maintenance of normal spinal reflexes. Drug-induced motor abnormalities such as stereotyped behaviour or excessive restlessness after

amphetamine and apomorphine are modified by lesions of the raphe nuclei (Costall and Naylor, 1974) and in an opposite direction by accumulation of endogenous 5-HT (Breese et al., 1974). Also if the brain 5-HT concentration is raised very rapidly by combining inhibition of MAO with either reserpine treatment (rabbits) or the administration of TRY (mice and rats) hyperactivity syndromes are observed (Brodie and Shore, 1957; Grahame-Smith, 1971a).

1.01.4 Behavioural responses

a) Convulsions

There are several examples of the ability of serotonergic neurons to 'normalise' behaviour, including responses to sensory stimuli. 5-HTP raises the threshold for audiogenic siezures in mice and also restores to normal the hypersensitivity to noise caused by reserpine (Boggan and Seiden, 1973). An increase or decrease in brain 5-HT concentrations respectively antagonises or facilitates electroconvulsive seizures and pentetrazole convulsions (Kilian and Frey, 1973). In keeping with this, the anti-convulsant effects of phenobarital against these two forms of convulsions are diminished by PCPA and enhanced by 5-HTP (Meyerson and Lavander, 1970). However, the anticonvulsant effect of diphenylhydration was unaffected by changing the cerebral 5-HT concentrations.

b) Aggression

Mouse killing was elicited by lesioning the raphe nuclei in

rats which previously remained indifferent to a mouse placed in their cage (Vergnes et al., 1973); similar effects were seen after treatment with PCPA (Sheard, 1969; Di Chiara et al., 1971). An important finding was that, however much PCPA was given, the aggressive behaviour was only seen in a percentage of the animals, showing the contributory rather than decisive role of serotonergic pathways.

Isolation-induced fighting in mice was found to be increased by lowering the 5-HT content of the brain with chloromethyl-amphetamine, and reduced by raising levels with 5-HTP and an inhibitor of peripheral decarboxylase.

c) Self-stimulation

Another instance of the role of 5-HT as an inhibitor of excesses in behaviour is shown with self-stimulation. Electrodes were implanted into the median forebrain bundle of rats and the rate of self-stimulation compared before and after either PCPA or 5,6-dihydroxytryptamine, both drugs used in doses which reduced the 5-HT content of the brain. Self-stimulation frequency was greatly enhanced by either drug at the time of lowest cerebral 5-HT concentrations (Poschel and Ninkeman,1971; Poschel et al.,1974).

(d) Sexual behaviour

If PCPA is given to adult males, male to male mounting is observed (Schillito,1969, 1970; Tagliamonte et al., 1969; Bond et al., 1972). The same phenomenon was seen when serotonergic neurons were damaged by 5,6-dihydroxytryptamine (Da Prada et al., 1972). It was also produced in cats

(Hoyland et al., 1970); after receiving PCPA males mounted indiscriminately other males and anoestrous females. All effects were temporarily abolished by small, non-sedative doses of 5-HTP. It may be inferred that serotonergic neurons inhibit inappropriate sexual behaviour and, by their restrictive influence, limit mounting to biologically desirable circumstances. In females the effect is in the same direction but far less pronounced: PCPA caused the occurrence of some aspects of oestrous behaviour in non-oestrous female cats (Hoyland et al., 1970) and an increase in lordosis and acceptance of the male in the rat (Everitt et al., 1974; Meyerson and Lewander, 1970).

1.01.5 Response to different stimuli

a) Pain perception

Tensen (1968) first drew attention to the fact that the analgesic effect of morphine in the rat was reduced by PCPA. A similar reduction was obtained by lesioning the raphe nuclei (Samarin, 1970). Electrical stimulation of the nucleus dorsalis raphe in the rat produces analgesia and this effect, too, is diminished by PCPA (Akil et al, 1972). Activation of serotonergic pathways thus reduces pain, and part, at least, of the action of analgesic drugs is exerted with the help of such pathways. An attempt was made (Vogt, 1974) to determine the location of the 5-HT containing fibres involved in this action of morphine. Rats were treated with a dose of 5,6-dihydroxytryptamine which damages the spinal serotonergic axons severely and for a period lasting several months,while

the effect on the cells of origin and all 5-HT containing neurons and on the ascending axons is slight (Baumgarten et al, 1972). This treatment only slightly reduced the analgesic potency of morphine, in contrast to the very pronounced effect of PCPA. The conclusion seems to be that the spinal serotonergic axons contribute to the analgesic effect of morphine, but are not the only ones involved.

b) Visual stimuli

The unusual density of serotonergic terminals in the superior colliculi of mammals prompted experiments on ways of activating these terminals. In vitro work (Kawai, 1970) had shown that a thin slice of guinea-pig superior colliculus suspended in an organ bath can be loaded with (^{3}H) 5-HT, and that the rate of release of radioactivity from the slice into the medium is accelerated by electrical stimulation of the optic tract. This raised the question whether, in vitro, stimuli would activate the 5-HT containing neurons terminating in the superior colliculus. Experiments were carried out on rabbits (Fukui and Vogt, 1975) which have crossed optic nerves so that it was possible to try and effect only one colliculus by exposing one eye to visual stimuli. A rise in the tissue concentration of the metabolite of 5-HT, 5-HIAA should indicate an increased turnover of 5-HT. A small, but significant increase in 5-HIAA content of the colliculus contralateral to the exposed eye was indeed found, whereas there were no differences in 5-HIAA content between other symmetrical parts of the brain taken from the right and left

hemispheres. This increased turnover of 5-HT only occurred when the visual stimuli were not stationery, but moved, and when the moving flashes were as varied and irregular as possible. These are precisely the conditions which also elicit the largest and most constant electrical evoked responses in the superior colliculi. One is led to the conclusion that conditions which keep the colliculi very active in discharging stimuli to other parts of the brain (e.g. the visual cortex) are equally favourable to the discharge of serotonergic neurons. Exogenous 5-HT being invariably an inhibitor of the discharge of collicular cells, this seems to be another instance of the damping activity of serotonergic neurons to be called into action by excessive afferent stimulation.

C) Other environmental stimuli

Enhanced reactivity to novel environmental stimuli accompany lesions in the raphe nuclei. An attempt was made in the rat (Srebro and Lorens, 1975) to relate this behaviour abnormality to individual nuclei. It was found that destruction of the dorsal raphe nuclei, in spite of causing a loss of 65% of forebrain 5-HT, elicited hardly any change in behaviour, whereas lesions in the median nucleus, while reducing forebrain 5-HT by only 36%, increased open field activity and responses to environmental changes.

Although it is not possible to give a common denominator to all physiological events in which serotonergic neurons

participate, their most typical role appears to be one of preventing meaningless or excessive responses of the organism to environmental stimuli. The damping influence is particularly clear in the exaggeration, by loss of 5-HT, of male sexual behaviour, convulsive seizures, aggression and frequency of self-stimulation. 5-HT subserves certain analgesic pathways and seems to prevent over-activity in part of the optic system. Sleep also requires the integrity of certain serotonergic pathways. In view of the obvious importance of these neurons to our well-being a far greater knowledge is required for detecting or correcting their failure. The following section discusses the evidence for possible abnormalities in serotonergic function which may result in certain illnesses in man.

1.11 5-HT in mental illness and neurological disease

1.11.1 5-HT and Affective Disorders

Defective brain 5-HT metabolism in depression was first indicated by the low 5-HIAA concentration in lumbar CSF originally reported over a decade ago (Ashcroft et al., 1966), and confirmed by most but not all subsequent studies (Goodwin and Past, 1975). Brain levels of 5-HT and 5-HIAA have also been reported to be lower in suicides than in control subjects (Pare et al., 1969; Asberg et al., 1976b).

Initially reports of this kind were taken to mean that defective brain 5-HT metabolism was solely responsible for some depressions in which case one might expect concentrations

of 5-HIAA to rise on recovery. As this does not occur, it was suggested that recovery may involve increased sensitivity of 5-HT receptors rather than increased 5-HT metabolism (Medical Research Council, 1972).

Even if brain TRY and 5-HT metabolism are deficient in depression it does not necessarily follow that these biochemical abnormalities have any causative role; they might merely be harmless secondary consequences of the illness. However, a number of trials have indicated that TRY has anti-depressive efficiency comparable to various other more commonly used drugs. Coppen and coworkers (1967) reported that TRY was as effective as electroconvulsive therapy (ECT). Although there is disagreement on this, animal work (Green et al., 1977) suggests that the beneficial effect of ECT could itself involve increased responsiveness of post synaptic 5-HT receptors. Other workers have not found it was a useful antidepressant in the majority of patients treated (Carroll et al., 1970; Murphy et al., 1974), despite evidence that platelet 5-HT stores and possibly central 5-HT turnover (estimated from the effect of probenecid on 5-HIAA levels in CSF) were increased (Murphy, 1972; Dunner and Goodwin, 1972).

While there is disagreement about the effectiveness of TRY as an anti-depressant, it is generally agreed that it potentiates certain other anti-depressants such as the 5-HT

re-uptake blocker chlorimipramine (Walinder et al.,1975) and MAO inhibitors (Pare, 1963; Glassman and Platman, 1969; Coppen et al., 1963; Gutierrez and Alino, 1971). These results suggest that the therapeutic effects involve 5-HT or at least an amine formed from TRY, destroyed by MAO and released into the synaptic cleft. Administration of 5-HTP as an anti-depressant has a theoretical advantage over TRY as it is more readily converted to 5-HT but it also has a disadvantage because not all of this 5-HT is in serotonergic neurons. Large doses have been claimed to be directly beneficial in bipolar depressives (Sano, 1972) or to potentiate the MAO inhibitor, nialamide, when given to a mixed group of endogenous depressives (Alino, 1976). Infusion of 5-HTP into normal subjects, previously given a peripheral decarboxylase inhibitor, caused a rapid elevation of mood but such a response was noted in only a small proportion of depressives (Graw et al., 1976).

Perhaps, the most persuasive evidence of a causal role for a 5-HT defect in some patients with depression is that those who are helped most by 5-HT precursors appear to be deficient in 5-HT and distinguishable as a group on clinical grounds from other patients who are resistant to 5-HT precursors (van Praag and Korf, 1971; Asberg et al., 1976a).

1.11.2 Indoleamines and Schizoprenia

The theories which implicate indoleamines in the etiology of schizophrenia are derived mainly through consideration of

the actions of psychotomimetic drugs and the relationship of some of these to indoleamine structures; the effects on the schizophrenic state of MAO inhibition and loading with TRY, methionine and cysteine; the neuropharmacological actions of phenothiazines in relation to their beneficial effect of schizophrenia; and, for monoamines in general, the implications of the 'paranoid schizoprenic synchrome' produced by chronic amphetamine usage.

However, the evidence to support these theories is in no way conclusive. Bradley et al., (1966) using microelectrophoretic studies suggested that chlorpromazine could exert its beneficial effects in schizoprenia by 5-HT receptor blockade. However, the ability of chlorpromazine to inhibit the actions of LSD, not only in animals but also in man, could be due to inhibition of dopaminergic pathways (Gordon, 1967). Green and Grahame-Smith (1974) proposed that dopaminergic pathways are probably involved distally in mediating the effects of the stimulation of certain 5-HT receptors which again cast in doubt the previous assertion that the phenothiazines act to block 5-HT receptors in vivo.

Metabolic evidence that there is either an abnormally high or an abnormally low rate of 5-HT turnover in schizophrenia is very unconvincing. Wyatt et al.,(1973a) have studied the effect on schizophrenia of administering 5-HT and a peripheral aromatic acid decarboxylase inhibitor. The drug combination was better than placebo in at least seven patients and in

two there was no difference; however, two patients became worse on the treatment.

The effects of 'precursor loading' in schizoprenia are also difficult to interpret. Pollin et al., (1961) administered various amino acids together with a MAO inhibitor to schizophrenic patients. TRY caused euphoria and a freeing of associations, while methionine caused increased anxiety, tension and motor activity, depression, increased hallucinatory activity and periods of disorientation with agitation. In general, these findings with methionine have been confirmed (Grahame-Smith, 1974; Smithies, 1974). A hypothesis for the worsening of the schizophrenic state was that methionine acts as a methyl donor, via S-adenosyl-methionine, to methylate an indoleamine and thus produce an endogenous psychotogen. The increasing evidence that N-methylated tetrahydrofolate may be the methyl donor for the indoleamine N-methyl transferase in brain weakens this hypothesis (this is further discussed in section 1.9.1). Several workers have looked for N-methylated indoleamine derivatives in schizophrenic and normal subjects but results are confusing (see section 1.9.1).

In summary, although there is no firm evidence of either a dysfunction in serotonergic pathways or the endogenous production of an indoleamine psychotogen in schizophrenia there are a number of leads to support continued investigation in this field.

1.11.3 5-HT and Mongolism

While it is well established that there are abnormalities in TRY metabolism in mongolism (Down's syndrome), there is still little information on the central indoleamine changes because of the difficulty of studying human brain metabolism and the lack of an adequate animal model. It has been shown by many workers that platelet 5-HT is below normal at all ages of mongolism (Posner et al, 1965; Tu and Zellweger, 1965; Boullin and O'Brien, 1971; Airaksinen, 1971). Urinary 5-HIAA excretion is increase in mongoloids (Airaksinen, 1971) but total 5-hydroxyindoles in CSF are normal. Studies on plasma TRY (Airaksinen and Airaksinen, 1972) showed normal fasting TRY but a smaller rise in mongoloids after TRY loading compared to normal children. The binding of TRY to plasma proteins in mongoloids is lower than normal (Airaksinen and Airaksinen, 1972) which might affect brain 5-HT synthesis.

A very different approach has been used to determine whether central 5-HT metabolism is abnormal in mongoloids by utilising the hypothesis that the platelet might be used as a model for the serotonergic neuron in the brain (Passonen, 1968; Pletscher, 1968). 5-HT uptake and binding in platelets from mongoloid and normal subjects was studied. Concentrations of endogenous 5-HT in mongoloid platelets were 25% of normal and net 5-HT accumulation was 50% of normal. Furthermore, efflux from 5-HT loaded platelets was much greater from mongoloid than from normal platelets. It was considered

that the low 5-HT in mongoloid platelets was due to defective transport and impaired binding, the latter resulting from the reduction of adenosine triphosphate (ATP) concentration also observed in these platelets (Boullin et al, 1969; Boullin and O'Brien, 1971). These authors also found decreased MAO activity in mongoloid platelets (Boullin and O'Brien, 1973).

Clearly, further work on the CNS is needed since there is probably little more to be gained from peripheral studies. Boullin (1975) measured 5-HT in the postmortem brain of one mongoloid child and found evidence of decreased 5-HT concentrations but this cannot be accepted as definite proof until there are further studies and appropriate controls. Many of the functions that 5-HT is suggested to control or modulate in the brain do not appear to be overtly impaired in mongoloids, and thus it remains to be determined whether central 5-HT metabolic abnormality has a primary role in the manifestations of Down's syndrome.

1.11.4 5-HT and Phenylketonuria

Phenylketonuria is an inborn error of metabolism in which there is a failure of the liver to hydroxylate phenylalanine to tyrosine. The plasma concentration of phenylalanine is therefore 20-40 times higher than normal and abnormal metabolites appear in the urine. The most obvious clinical result is severe mental retardation.

The demonstration that serum 5-HT and urinary 5-HIAA concentrations were decreased in phenylketonuria (Pare et al., 1957, 1959) led to animal studies on indoleamine changes in animals fed a high - phenylalanine diet. It appeared that most changes in both peripheral and central 5-HT were probably due to an effect on TRY transport. Yarbro and Anderson (1966), for example, found defects in TRY uptake from the gut of phenylketonurics, this change being reversed by giving a low-phenylalanine diet. Similar inhibition probably inhibits uptake of TRY into brain slices (Grahame-Smith, 1968; Green and Curzon, 1970) and synaptosomes Grahame-Smith, 1968). Brain 5-HT was decreased in rats given a high-phenylalanine diet, but there was no correlation in their performance in various tests and the brain 5-HT concentrations (Yuwiler and Louttit, 1961). Nor was there any improvement in phenylketonuric children given 5-HTP or MAO inhibitors to increase brain 5-HT, and it is now generally considered that the changes in central 5-HT metabolism in phenylketonurics are not responsible for the mental impairment or pathogenesis of the disease.

Considering the evidence presented in this introduction for the physiological role of 5-HT in the CNS and its possible involvement in certain disorders, it is important to understand the controlling mechanisms of the functional activity of serotonergic neurons. It is hoped that by studying the possible biochemical factors controlling the circadian variation in 5-HT concentrations in the rat brain, more information on this subject may be gained.

CHAPTER TWO

Cabinet characterisation and animal maintenance

2.1 Introduction

Within any laboratory or animal house, the normal environmental conditions vary daily and annually with respect particularly to lighting, temperature and noise levels. In any animal experiment it is necessary to control these variables and it is especially important to maintain a controlled environment when estimating circadian fluctuations as there is evidence to suggest that 5-HT levels and metabolism in the brain are susceptible to environmental changes (Hauty and Adams, 1966a, 1966b; Schwartz and Aghajanian, 1969; Thierry et al., 1968). For these reasons, specially constructed cabinets were used to house the rats, (described in Section 2.2). These also had time clocks fitted to enable the experimenter to work during the normal period of a day and thus to avoid circadian changes in the performance of the experimenter.

2.2 Cabinet Construction

Fig. 6 is a photograph of the cabinets used in these experiments. The cabinets were made from 6/10 in. blockboard and the dimensions of each are 18 x 24 x 48 ins. Lighting was supplied to each cabinet by a 12 in. miniature fluorescent fitting with an 8 watt, warm white tube (Thorn,L.J. S1008 H). To prevent overheating the choke was removed from each fitting and reassembled outside the cabinet. Each light was connected through a time switch (Sangamo Type 5254-1-171). To ensure light proofing, ½ in. plastic foam strips were

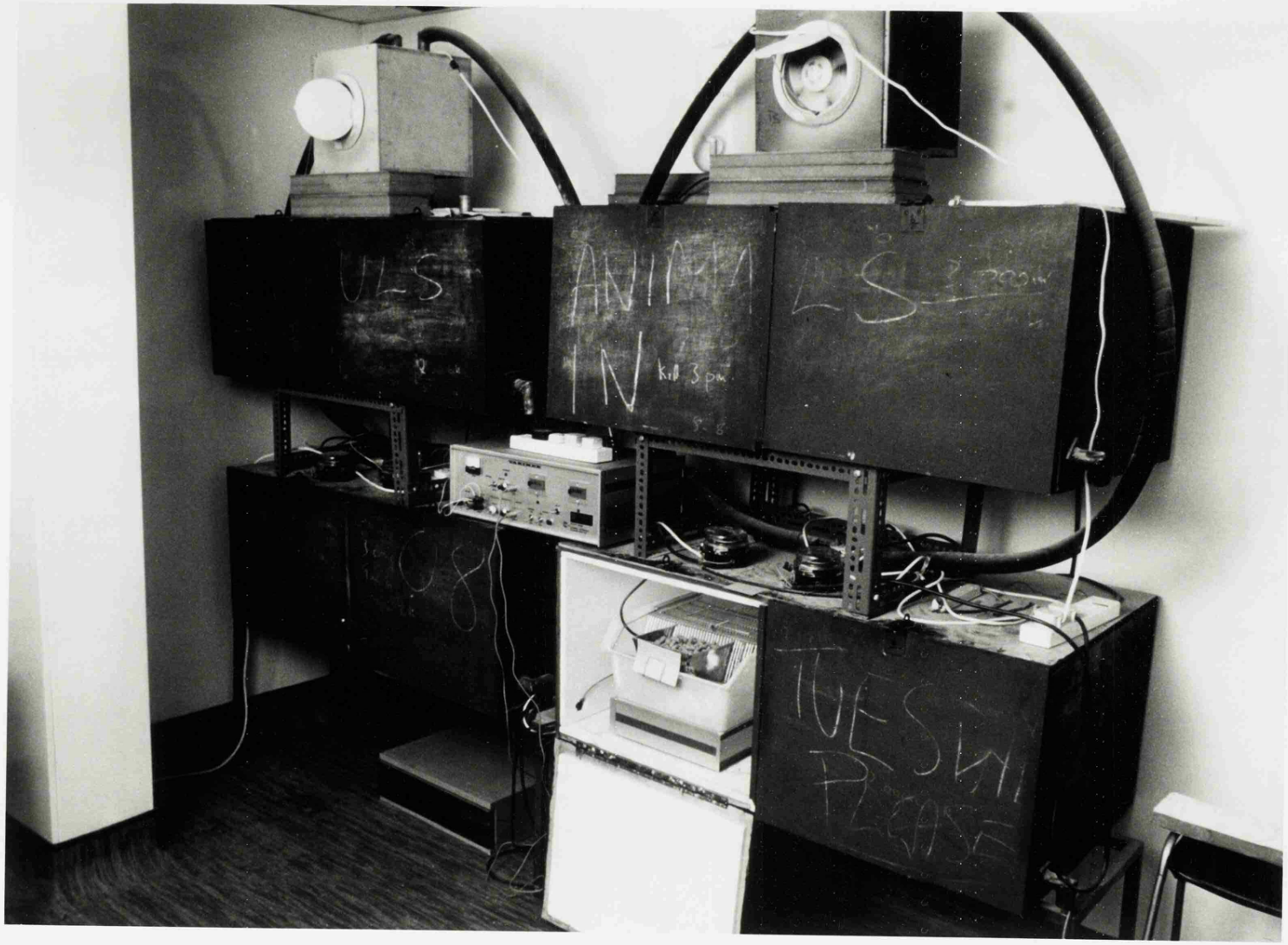


Fig. 6

glued to the perimeter of the inner door face and its adjoining surface. Sound insulation of each cabinet was improved by a lining of ½ in. polystyrene sheeting. The problem of ventilating and maintaining the environment at a constant temperature was overcome by using domestic extractor fans (Xpelair FXC 6) to draw a constant flow of air through the cabinets. One fan, which was capable of removing 1,200 cu. ft. of air per hour served four cabinets, to which it was connected through triple gauge 1 1/4 in. bore rubber tubing. The intake was first passed through an insulated chamber containing a 300 watt infra red bulb. This was activated by a thermostat inside the cabinet so that in the event of a fall in temperature inside the cabinet, the temperature of the air input was automatically increased. A constant watering device was fitted and by using extra large food hoppers on the cages, animals (in groups of five or three) could be left undisturbed for up to fourteen days.

The characterisation of the cabinets i.e. light proofing, sound proofing and temperature variation had been previously investigated (Hillier et al., 1973) and found completely satisfactory. For this reason the work was not repeated for this thesis.

2.3 Choice of animal

Male CFY rats were used throughout the experiments described. Rats were chosen for the following reasons:-

(a) Economy, availability, ease of maintenance, handling and use.

(b) Strains of this species are the most standardised animals available to the laboratory investigator due to inbreeding and also the most widely studied in pharmacology (Woodward, 1965) thus providing a balance between control of variation and applicability of results.

2.4 Maintenance of animals

Male CFY rats (100-120g) were kept in groups of five (biochemical analysis) or three (behavioural work), with one group per cabinet. The rats had access to food (Oxoid Breeding Diet) and water ad libitum and could remain undisturbed for up to fourteen days.

As stated previously, experiments were performed during the normal working period of the operator which meant that many of the rats had to undergo a phase shift upon entry to the cabinets. At least ten days were therefore allowed for re-adjustment and acclimatisation for all groups of animals (Davies et al., 1974). There was no recognisable difference between groups of animals maintained on a 'normal' lighting regimen and those on the reversed schedule. Weight gain, weight of food consumed and volume of fluid intake were all

similar.

The animals were always maintained on a 12 hour light: 12 hour dark cycle and temperature was maintained at 24 $\pm$ 1.5^{o}C throughout. When the animals had to be killed or injected during a dark phase, the laboratory was illuminated only by a photographic red safety lamp, since red light has been shown to have no significant effect on circadian variation (McGuire et al., 1973).

CHAPTER THREE

The 24-hour variation in total, 'free' and bound TRY in the rat brain

3.1 Introduction

TRY is one of the essential amino acids in the body. It is also the precursor for the putative neurotransmitter, 5-HT Many factors influence the level of TRY in the blood and brain and the relevance of these will be discussed in terms of affecting 5-HT synthesis in serotonergic neurons in the brain.

The daily flux of amino acids in the body is extensive. About 300g of protein is synthesised each day in an adult man. This requires the uptake and release of 150g of essential amino acids, yet the minimum dietary requirement for essential amino acids is only 6g while the customary diet contains 45g (Munro,1972b). This indicates considerable and efficient recycling of the essential amino acids released by protein breakdown. One reason for this is the restriction of key catabolic enzymes of 7 of the essential amino-acids to the liver (Miller, 1962). Consequently, considerable amounts can be reused for protein synthesis within cells providing these amino-acids do not leak into the blood in large amounts and reach the liver.

After a meal rich in protein, the amount of free amino acids in the portal vein can rise considerably during the absorptive phase, whereas their concentration in the systemic circulation changes much less. Studies on dogs having cannulae in the blood supply to and from the liver show that this is due to the enormous capacity of the liver to remove amino acids from the portal vein and thus regulate their flow into the systemic circulation (Elwyn, 1970).

In humans, plasma TRY and most other amino acids exhibit characteristic fluctuations every day reaching a peak mid-afternoon if the subject is on a standard mealtime schedule (Wurtman et al.,1968; Wurtman,1970). A similar rhythm was subsequently noted in rats for plasma TRY but the peak was reached in the dark period (Fernstrom et al.,1971). This phenomenon was attributed to the rat's tendency to consume most of its food during the hours of darkness.

Approximately 70% of TRY is oxidised by tryptophan pyrrolase in the liver to formylkynurenine. This enzyme displays a 24-hour variation in activity and it has been suggested this could control the rhythm in plasma TRY. (Rapoport et al.,1966). Young and Munro (1973) showed that TRY levels in plasma and the activity of liver tryptophan pyrrolase increased only when basal dietary TRY requirements were exceeded indicating an adaptation of the enzyme after a meal. However, Ross et al.,(1973) found that whereas tyrosine aminotransferase was rapidly activated by protein consumption, tryptophan pyrrolase was unaffected. Nonetheless, it was noticed that pyrrolase activity reached a peak around the time of day that the rats ate. Thus, while the enzyme may not be activitated by TRY it seemed to be active at the appropriate time of day. Intact adrenocortical function is required for expression of a normal rhythm of both pyrrolase and TRY although an altered and less apparent rhythm persists in the adrenalectomised state (Rapoport et al.,1966) indicating that enzyme activity is under hormonal control. Joseph et al.,

(1976) showed that the amount of $^{14}CO_2$ exhaled quadrupled after injection of a tracer dose of {2-^{14}C}TRY if 5mg/kg cortisol had been injected 150 mins previously. This increase was proportionately at least as great as the increase in kynurenine synthesis from TRY by liver tissue in vitro.

The mere existence of rhythms in plasma concentrations of amino acids did not establish that such variations were of any consequence physiologically. The plasma rhythms in humans persisted for two weeks in volunteers who ate essentially no protein (Wurtman et al.,1968) yet disappeared in subjects on a total fast (Marliss et al.,1970); this finding suggests the rhythms are not truly circadian or of endogenous origin and that the most important factor in their genesis is nutritional. The significance of these rhythms was further explored by determining whether the metabolic fate of an amino acid could be influenced by daily fluctuations in its plasma level. Intraperitoneal injections of TRY which change plasma TRY concentrations by an extent smaller than the circadian variation were found to produce a significant rise in brain 5-HT levels (Fernstrom et al.,1971). Experiments were then carried out to determine whether physiological decreases in plasma TRY could decrease amine content. Insulin reduces almost all other amino acid levels in plasma by enhancing their uptake into skeletal muscle (Wool, 1965). However, Fernstrom and Wurtman (1972a) found insulin increased plasma TRY levels by 30-40% and also

increased brain TRY and 5-HT levels whether injected or by secretion elicited by a carbohydrate diet fed to fasted rats. It was predicted that a diet of carbohydrate plus protein would raise 5-HT levels still further (Fernstrom and Wurtman,1972b). This was not the case; plasma TRY levels rose sharply but brain TRY and 5-HT levels were unaltered. If the amount of protein in the meal was increased beyond the 18-22% contained in most commercial rat chows, brain TRY and 5-HT could even fall.

Other investigators, using brain slices (Blasberg and Lajtha , 1965) or animals treated with pharmacological doses of individual amino acids (Guroff and Udenfriend,1962) had shown that groups of amino acids (e.g. neutral, acidic and basic) are transported into the brain by specific carrier systems, and that within a group, the member amino acids compete with each other for common transport sites. Fernstrom and Wurtman explained their results in terms of the ratio of plasma TRY to the other neutral amino acids (tyrosine, leucine, phenylalanine, valine and isoleucine). Since carbohydrate ingestion elicits insulin secretion, it simultaneously raises plasma TRY and lowers the concentration of the competing amino acids leading to elevations in brain TRY. In contrast, virtually all natural proteins contain less than 1.5% TRY but more than 25% of their total mass derives from the five competing amino acids (tyrosine, leucine, phenylalanine, valine, and isoleucine). Moreover tryptophan pyrrolase catabolises

a considerable fraction of TRY entering the liver via the portal vein while little or no metabolism of the branch-chained amino acids (leucine, isoleucine and valine) occurs in the liver. Hence, the consumption of any protein causes proportionately greater increases in the plasma concentration of tryptophan's competitors than in that of TRY itself. This was verified when the same diet minus the five neutral amino acids increased brain TRY and 5-HT. Removal of aspartate and glutamate, 2 amino acids using a different amino acid transport system from TRY failed to alter brain TRY and 5-HT concentrations. These results show that brain TRY and 5-HT levels do not simply reflect those of plasma TRY but depend also upon the plasma concentrations of the other neutral amino acids.

However, these studies measured total plasma TRY and did not take into consideration the unusual property of TRY, its ability to bind to serum albumin. About 10-20% circulates as the free amino acid (McMenamy et al., 1957) . No other amino acid binds appreciably to plasma proteins. Because binding in general implies storage, several investigators have suggested that the plasma free TRY is the functionally important pool in determining the availability of circulating TRY to brain and other tissues (Knott and Curzon, 1972). This hypothesis was tested by a study of the effects of 2 environmental manipulations- immobilisation stress and food deprivation (Curzon et al., 1972). Both treatments

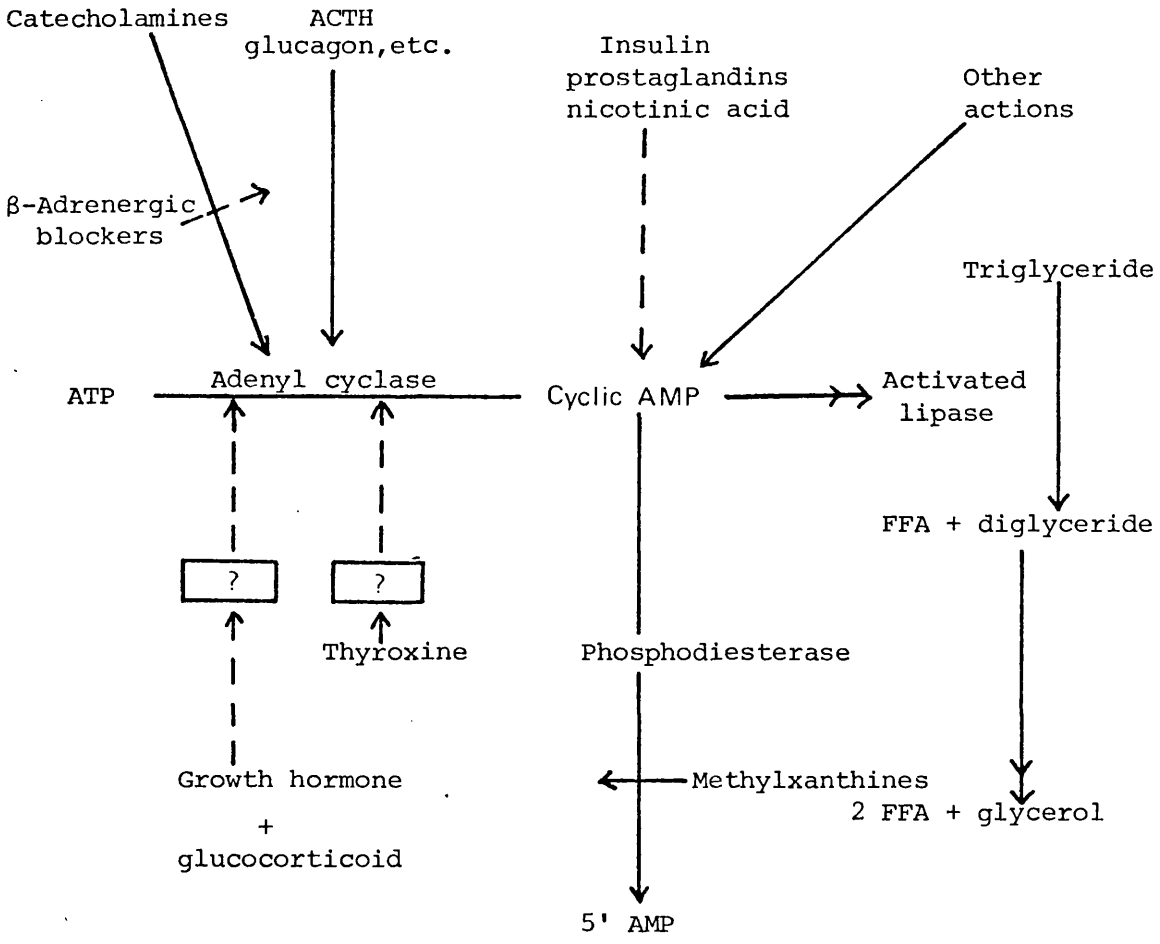


Fig. 7 Effects of drugs and hormones on lipolysis (from Robison et al., 1971): FFA=NEFA.

significantly increased brain TRY and 5-HT turnover as indicated by increased 5-HIAA but did not increase brain amino acids generally (Knott et al., 1973). It seemed that brain TRY changes were generated by changes in plasma. However, brain TRY increased while plasma TRY levels remained constant. This anomaly was clarified when both total and free plasma TRY were determined. It was found that the increase of brain TRY was associated with a shift in equilibrium between free and bound TRY so that the free material increased. One way in which the equilibrium can be altered is by non-esterified fatty acids (NEFA), which are almost completely bound to albumin. When fatty acid concentrations rise, plasma TRY is freed and made more available to the brain. Food deprivation and emotional stress are known to increase plasma NEFA (Dole, 1956; Mallov and Witt, 1961) and free TRY levels rise if fatty acids e.g. linoelic, oleic and palmitic acid are added to plasma in physiological concentrations (Curzon et al., 1973). These results suggest that many agents which affect lipolysis and plasma NEFA concentrations (Fig. 7) by acting on fat cell cyclic AMP could influence the disposition of plasma TRY and thus 5-HT turnover in the brain. Catecholamines certainly alter plasma NEFA levels. Havel and Goldfien (1959) demonstrated that NA increased plasma NEFA concentrations presumably through an action on β receptors since isoprenaline had the same effect.

Both these treatments raised free TRY levels as did injection of L-dihydroxyphenylalanine (L-DOPA) at high doses (Curzon and Knott, 1974). Previous reports had stated that intravenous infusion of L-DOPA into dogs increased plasma NEFA (Rivera-Calimlin and Bianchine, 1972) and that L-DOPA injection into rats increased brain TRY concentrations (Weiss et al., 1971) The phosphodiesterase inhibitor aminophylline also increased plasma NEFA levels and therefore as expected, increased plasma free TRY and brain 5-HT turnover. While nicotinic acid only caused a small plasma NEFA decrease in fed rats, it did abolish the rise in NEFA levels that occurred during food deprivation. It thus almost totally abolished the changes in plasma and brain TRY and brain 5-HT turnover that occurred during deprivation (Curzon and Knott, 1974).

The effect of drugs have also been used to investigate whether those which increase serum free TRY increase brain TRY and 5-HT levels and vice versa. Salicylate, probenecid and clofibrate which are able to release TRY from its binding to serum albumin (McArthur and Dawkins, 1969) are capable of increasing brain TRY levels (Gessa and Tagliamonte, 1974). Of the treatments that stimulate synthesis of 5-HT for instance D-amphetamine, reserpine, lithium carbonate, adenosine 3'5' monophosphate (cyclic AMP) and electroconvulsive shock only D-amphetamine and lithium significantly increased the amount of serum free TRY and neither compound released TRY from its protein binding when added in vitro. It would

appear that changes in free plasma TRY affect brain TRY and 5-HT levels rather than the opposite way round. All these studies would suggest that changes in free plasma TRY levels reflect similar changes in brain TRY and 5-HT levels. However, an experiment using streptozotoxin, a drug which destroys the pancreatic cells that secrete insulin(Arison et al.,1967) thereby decreasing plasma insulin concentrations, raised plasma NEFA levels and free TRY concentrations but brain TRY did not alter. (Fernando et al.,1976). This implied that lowered insulin levels resulted in a decrease in the effectiveness of TRY uptake into the brain as the level of competing amino acids was unaltered. Another piece of evidence supports the conclusion that both plasma TRY binding and competing amino acids influence brain TRY concentration. Etienne et al.,(1976) examined the uptake of TRY into the brain using the technique of Oldendorf (1971). The uptake of radioactive TRY injected into the carotid artery was diminished both by addition of albumin and by addition of competing amino acids. Addition of albumin did not alter uptake of tyrosine, which is not albumin bound.

As stated there are many factors which can influence free TRY availability to the brain such as plasma NEFA concentration, the concentration of neutral amino acids competing for uptake into the brain, responses at α and β receptors and the activity of tryptophan pyrrolase. Much of the work has

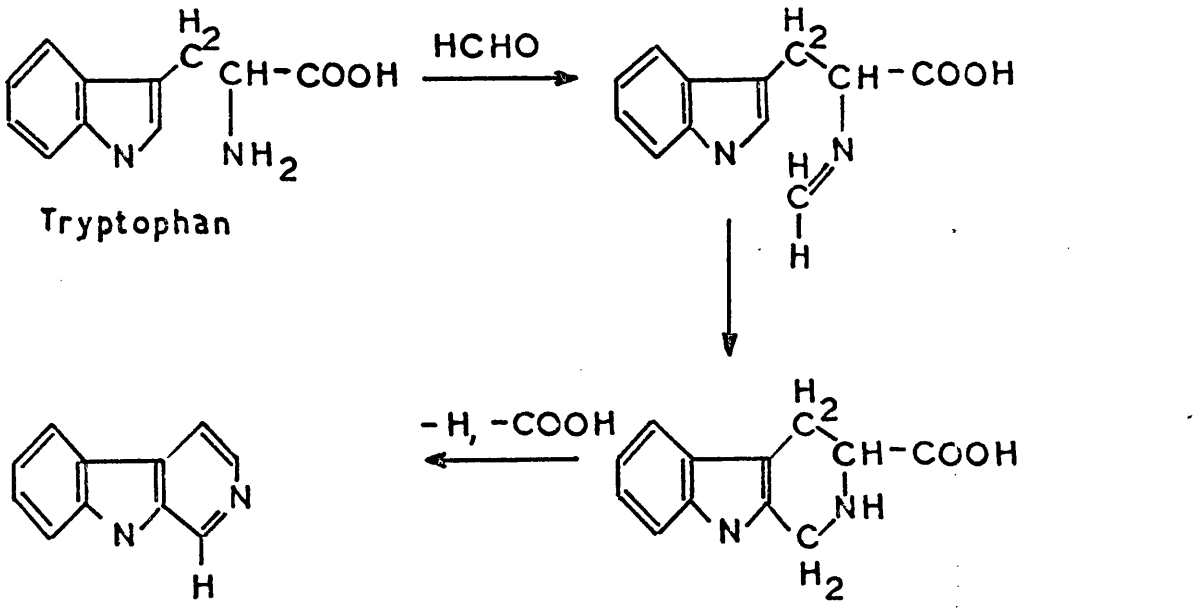
involved pharmacological manipulations of TRY levels in the blood, which depending on the circumstances i.e. the relative contribution of the factors mentioned previously, may lead to an increase in brain TRY. In situations when brain TRY levels do rise, in general, there is a concurrent effect upon 5-HT and/or 5-HIAA concentrations. This is thought to be due to the fact that tryptophan hydroxylase, the rate-limiting step in the synthetic pathway, is unsaturated with substrate under normal physiological conditions (this is discussed further in Chapter 5). The possibility of the regulation of 5-HT synthesis via brain TRY concentrations under normal physiological conditions, however, must be questioned when considering the circadian rhythms of 5-HT and TRY concentrations in the rat brain. Serum free TRY and brain TRY levels reach their peak values in the dark period (Fernstrom and Wurtman,1971; Hery et al., 1972)which corresponds to the animals' peak feeding activity while 5-HT concentrations are highest in the light period (discussed in Chapter 1). This 180° phase difference would be extremely difficult to explain if the assumption that brain TRY levels controlled 5-HT synthesis were true, particularly as previous findings in our laboratories had shown that the 24-hour variation in 5-HIAA levels closely followed that for 5-HT i.e. 5-HT levels were not low in the dark phase because turnover was high.

In these circumstances it appeared that endogenous TRY concentrations do not control the 24-hour variation of 5-HT

levels. However, the TRY estimations for the circadian variation were of total brain TRY levels. I thought as serum free TRY levels invariably reflect brain TRY levels perhaps the same criterion is relevant in that only 'free' TRY in the brain is the source of substrate for tryptophan hydroxylase. For this reason an experiment was devised to measure the levels of total, bound and 'free' TRY in the rat brain over 24 hours.

3.2 Method

The assay used to determine levels of total, protein bound and 'free' TRY in the rat brain involves the formation of the fluorophore norharman, from TRY by condensation with formaldehyde and then oxidative decarboxylation with ferric chloride ($Fe\ Cl_3$). This method is an adaptation



Tryptophan

Norharman

of that of Denkla and Dewey (1966) who measured TRY

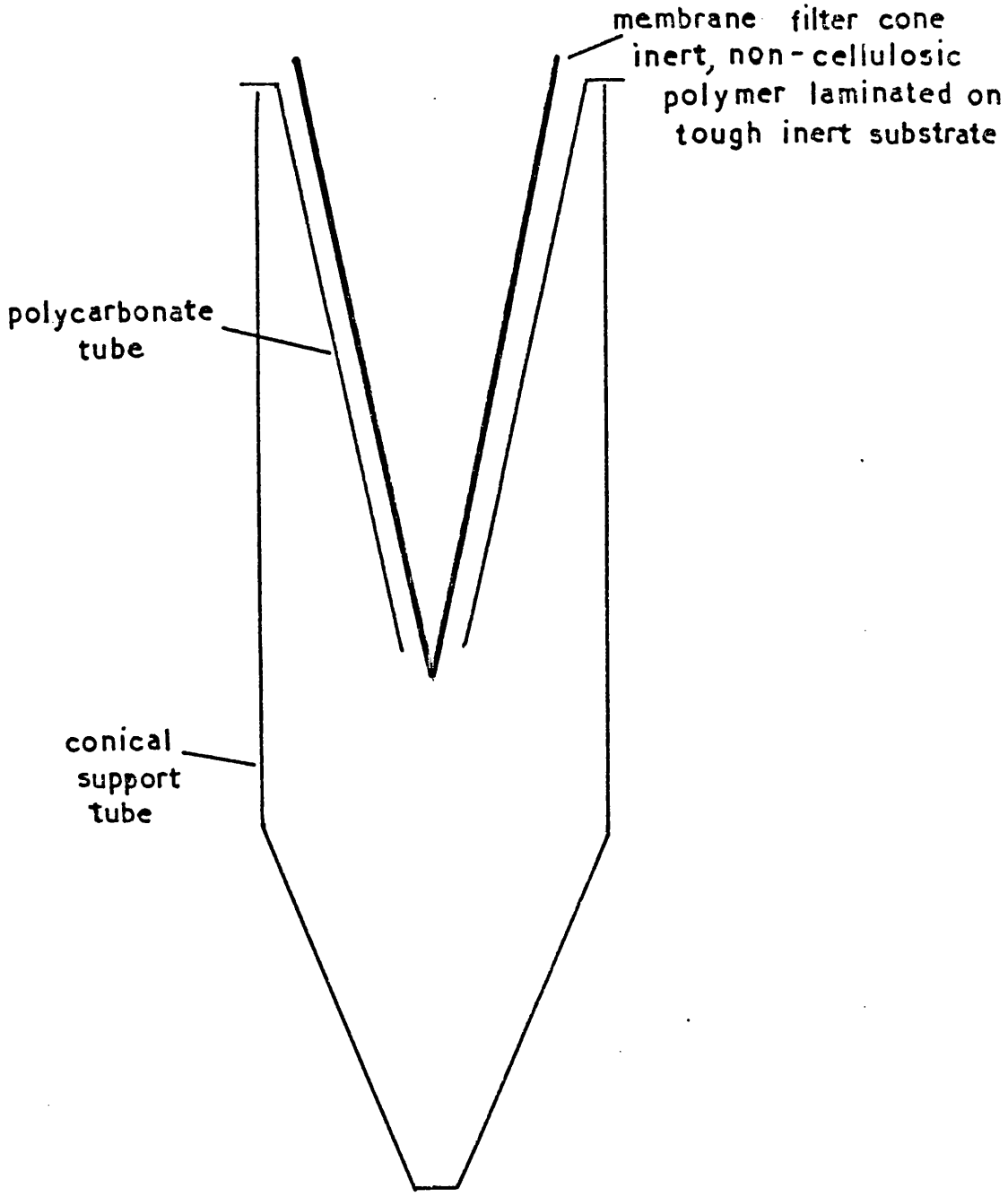


FIG. 8

Diagram of the apparatus used to prepare the ultrafiltrate samples

levels in plasma, urine and liver.

3.2.1 Preparation of samples

Male CFY rats (180-220g) were killed by decapitation, the brains rapidly removed and the pineal glands discarded. After weighing, each brain was homogenised in 3mls ice cold 0.9% saline (pH 7.4), and each homogenate divided in two. To one fraction, 1ml of 75% trichloroacetic acid (TCA) was added and after thorough mixing this sample was used to measure total brain TRY levels. 1ml of 0.9% saline was added to the remaining fraction and this was used for 'free' TRY estimations. This procedure was repeated 4 times but the volume of the homogenisation medium (8mls,13mls, 18mls and 23mls) was increased each time, thus altering the equilibrium between the 'free' and bound TRY. The ratio between each successive increase in homogenisation medium volume was inversely proportional to the final concentration of TCA.

3.2.2 Estimation of 'free' TRY in rat brain.

The samples were centrifuged for 15 mins at 1000g at $4^{o}C$, the supernatants decanted off and 1.5ml aliquots pipetted into centriflo CF50A filter cores (Fig. 8) previously prepared by soaking for 1 hour in distilled water. These filters have >95% retention for molecules above 50,000 MW, thus protein will be retained. The cones plus their supports were centrifuged for 30 mins at 1,000g at room temperature (excessive g force will cause protein leakage and may burst

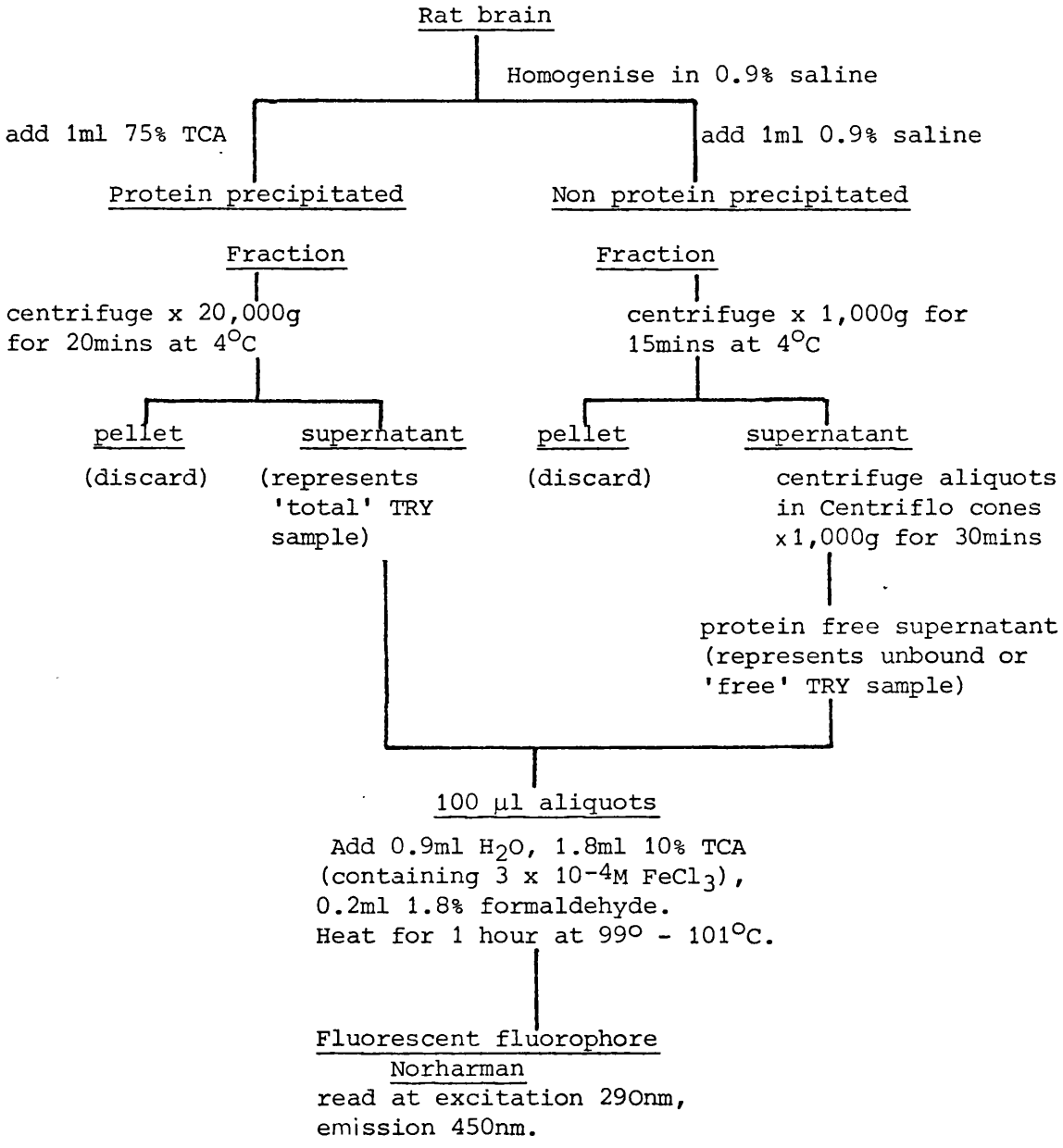


Fig. 9 Summary of the method used to measure total and 'free' TRY in the rat brain.

the filter). 100µl aliquots of the ultrafiltrates were added to 0.9ml distilled water, 1.8ml 10% TCA (w/v) containing 3 x 10^{-4}M $FeCl_3$ and 0.2ml 1.8% formaldehyde in a test tube and the contents mixed. After stoppering, the tubes were placed in a water bath for 1 hour at 99°C to 101°C. Temperatures below 99°C seriously affected the yield. Following the reaction, the tubes were cooled to room temperature and the product read at excitation and emission wavelengths of 290nm and 450nm respectively in a quartz cuvette in an Aminco-Bowman spectrophotofluorimeter. Since the change in fluorescence of norharman is negligible between 22° to 30°C, no special cooling is necessary. An internal standard of 100ng of TRY was prepared for each sample and reagent blanks were prepared by replacing the 100 µl sample with 100µl 0.9% saline.

3.2.3 Estimation of total TRY in the rat brain

The protein-precipitated samples were centrifuged at 20,000g for 20 mins at 4°C. 100µl aliquots of the supernatants were made up to 1ml with distilled water and mixed with 1.8ml 10% TCA (w/v) containing 3 x 10^{-4}M $FeCl_3$ and 0.2ml 1.8% formaldehyde. Resulting solutions were heated at 99°C to 101°C for 1 hour, cooled to room temperature and read at excitation 290nm and emission 450nm. Internal standards and blanks were prepared as described in section 3.2.2.

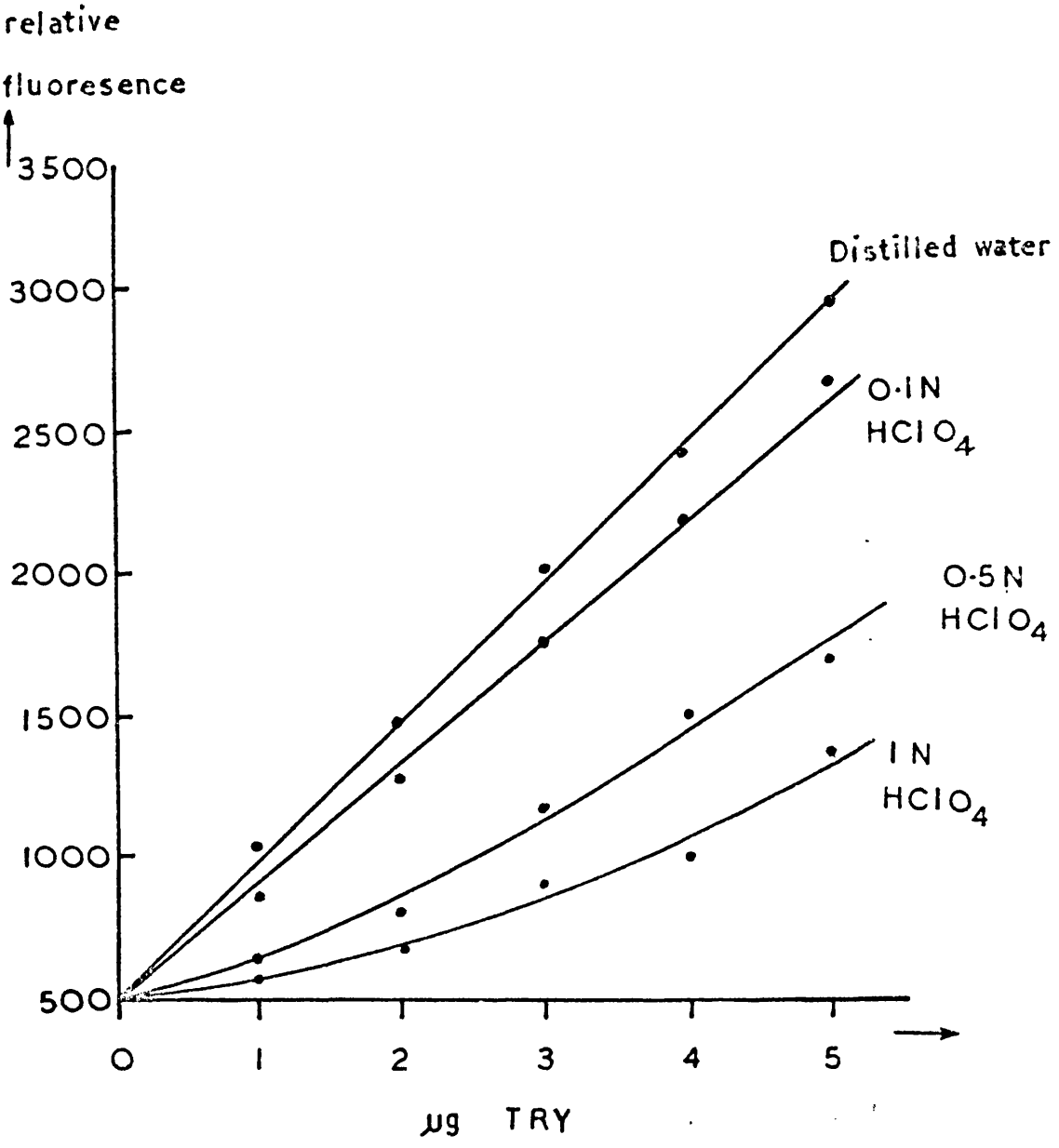


Fig.10 *The effect of increasing the concentration of perchloric acid (0.1N, 0.5N, 1N) on the relative fluorescence of TRY standards*

This standard method was arrived at after experiments were carried out to determine the optimal conditions for the assay. These experiments and their results are described in section 3.3. This method then formed the basic procedure for the determination of total, bound and 'free' TRY in the rat brain over 24 hours.

3.2.4 The 24-hour variation of total, bound and 'free' TRY in the rat brain.

Male CFY rats (180-220g) were maintained on a 12 hour light: 12 hour dark cycle for 14 days as described in Chapter 2 and then killed by decapitation at 4 hourly intervals. The brains were removed and prepared as described in section 3.2.1 with one exception i.e. the homogenisation medium volume was kept constant at 13ml. 'Free' and total TRY were determined as described in sections 3.2.2 and 3.2.3 and bound TRY values were calculated as the difference between the total and 'free' TRY concentrations.

3.3 Results

Expt. 1 Standard curve for L-TRY in the presence of increasing concentrations of perchloric acid.

Standards (1-5 µg TRY in a final volume of 4mls) were heated for one hour at 99° to 101°C with 0.1ml 85% TCA (w/v) containing 10^{-2}M $FeCl_3$ and 0.1ml 18% formaldehyde, and read at excitation 370nm and emission 450nm. Fig. 10 shows when the standards are assayed in increasing concentrations of

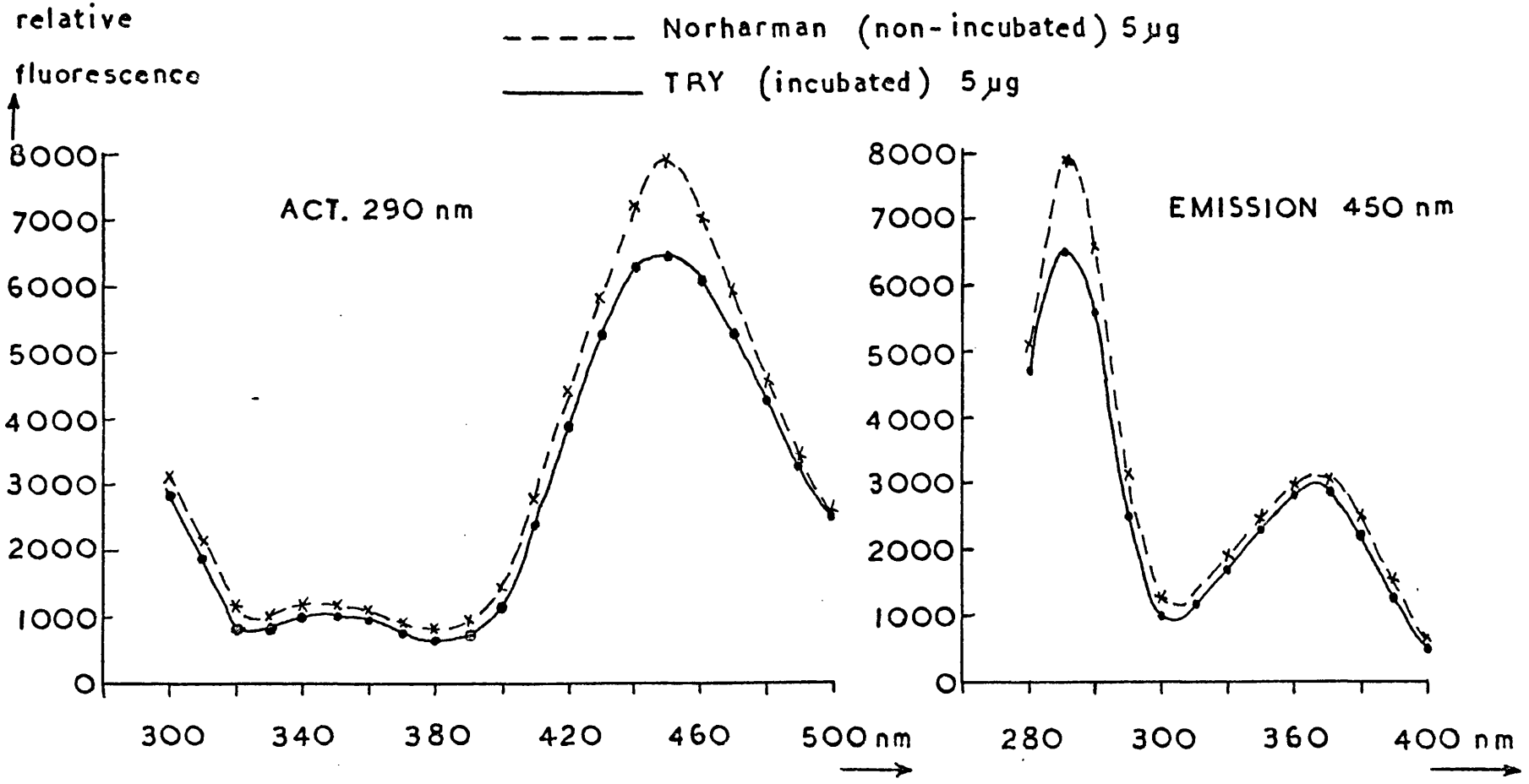


Figure 11 The excitation spectra of norharman and the TRY product

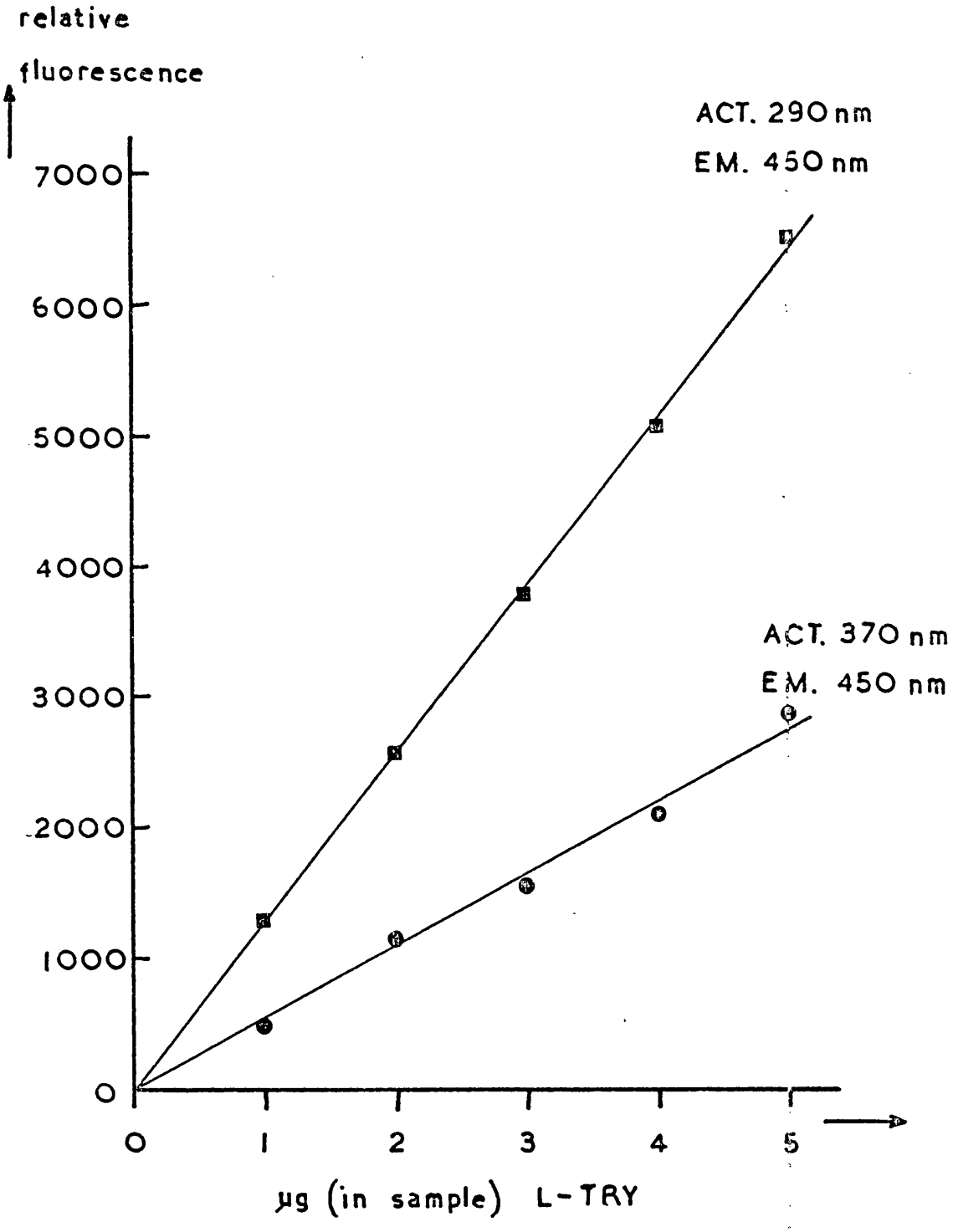


Figure 12 The effect of TCA (10%) on the relative fluorescence of TRY standards: emission 450nm; activation 290nm, 370nm.

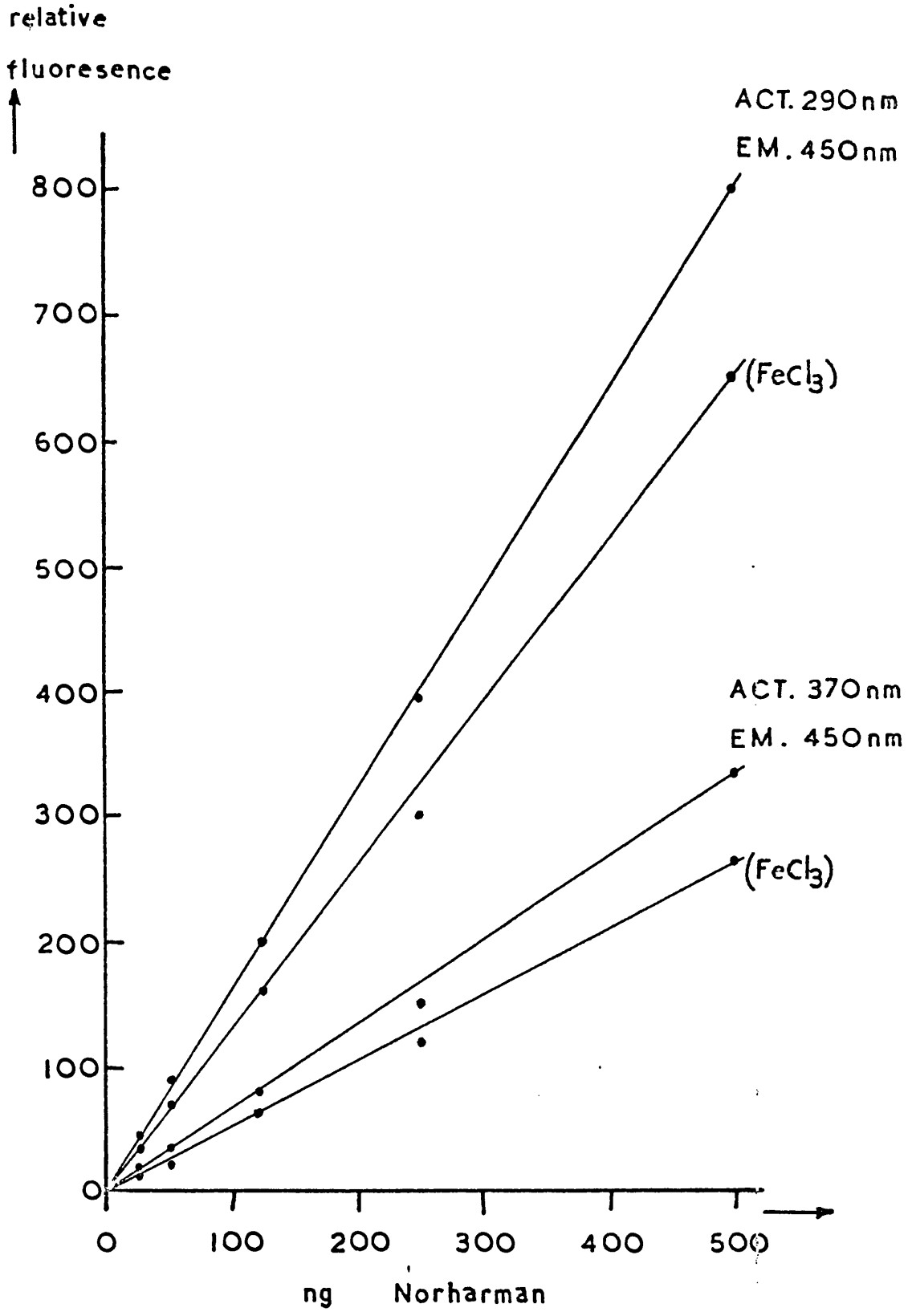


Figure 13 *The effect of 2.5 x 10^{-4}M $FeCl_3$ (final conc^n) on the relative fluorescence of norharman (in 0.1N HCl): emission 450nm; activation 290nm, 370nm.*

perchloric acid instead of distilled water, the relative fluorescence values are decreased.

Expt. 2 Standard curve for L-TRY in the presence of TCA. Standard L-TRY solutions of 10, 20, 30, 40, 50 µg/ml were prepared from which 100µl samples were taken. The samples were heated for 1 hour at 99° to 101°C with 0.2ml 1.8% formaldehyde and 1.8ml 10% TCA containing 3×10^{-4}M $FeCl_3$. After scanning 5µg norharman dissolved in 3ml 0.1N hydrochloric acid (non-incubated) and the 5µg standard (Fig.11), the samples were read at excitation 290nm and 370nm and emission 450nm. Fig.12 shows that relative fluorescence readings at excitation 290nm are greater than those at excitation 370nm. The standard curves are also linear in the presence of TCA unlike those in perchloric acid.

Expt. 3 The effect of 2.5×10^{-4}M $FeCl_3$ (final concentration) on the relative fluorescence of norharman.
50µl of 10^{-2}M $FeCl_3$ or 0.1N hydrochloric acid was added to 2mls of 0.1N hydrochloric acid containing 0-50µl of solution of norharman. The samples were read at excitation 290nm and 370nm and emission 450nm. Fig.13 shows unreacted $FeCl_3$ has a quenching effect on the standard curve of approximately 20%.

Expt. 4 The effect of increasing the volume of ultrafiltrate as a function of relative fluorescence.
The ultrafiltrate was prepared as described in section 3.2.2 (the brain was homogenised in 8mls of ice cold 0.9% saline) 0-500µl samples of ultrafiltrate were made up to 1ml with distilled water heated at 99° to 101°C for 1 hour with 0.2ml 1.8% formaldehyde

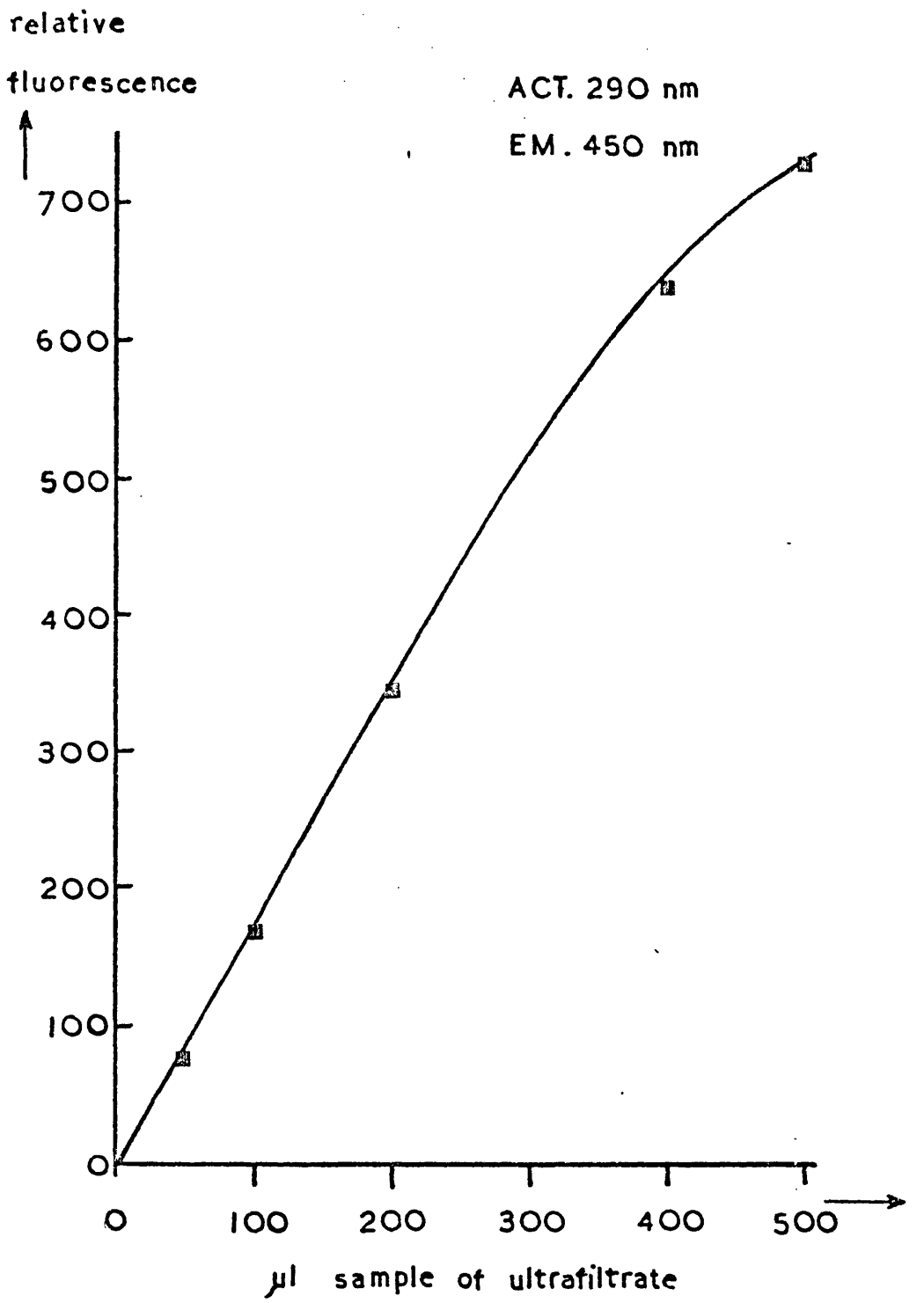


Figure 14 Estimation of TRY present in increasing volumes of ultrafiltrate: emission 450nm, activation 290nm

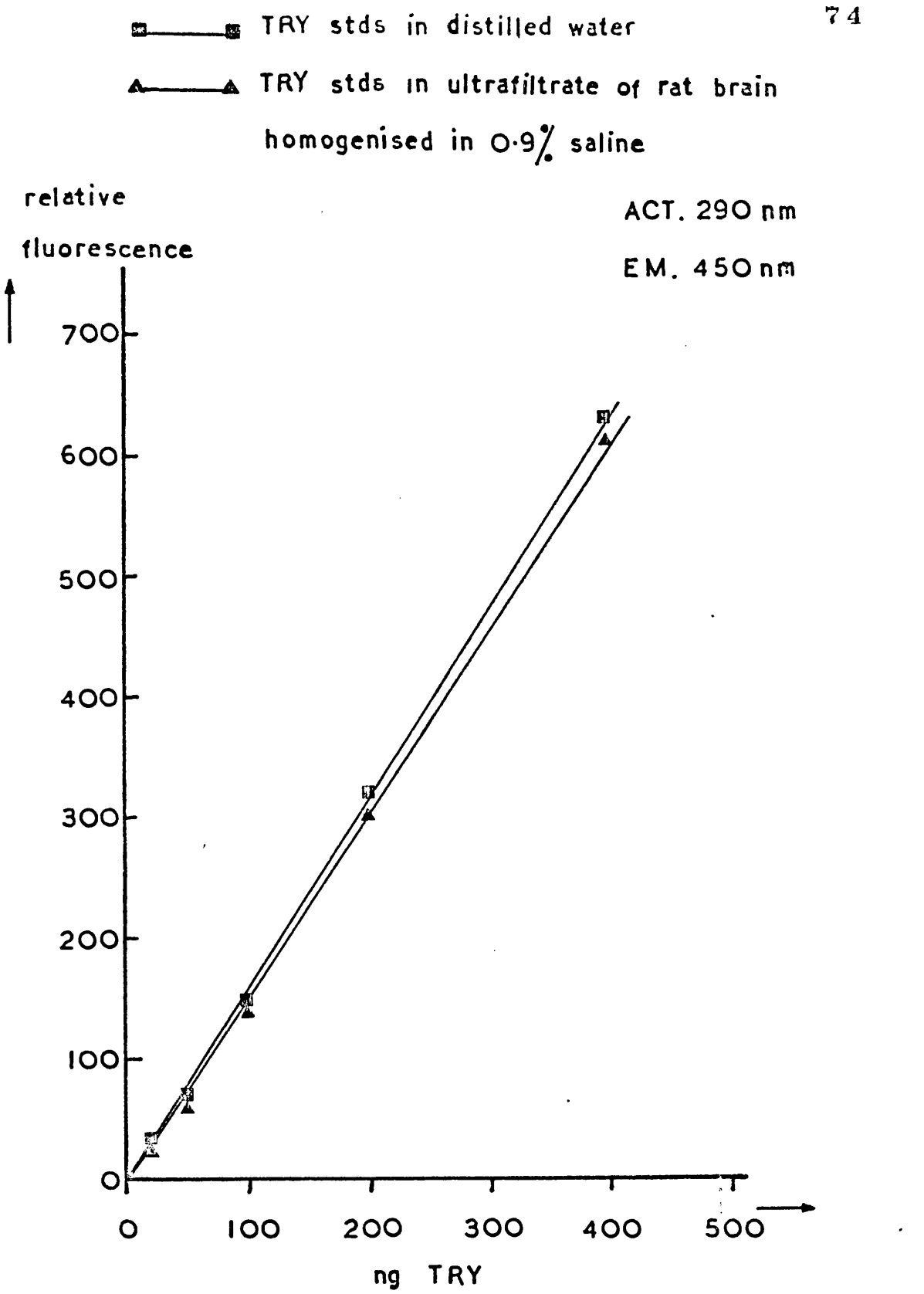


Figure 15 The relative fluorescence of TRY standards measured in the presence of an ultrafiltrate preparation (300µl): emission 450nm, activation 290nm

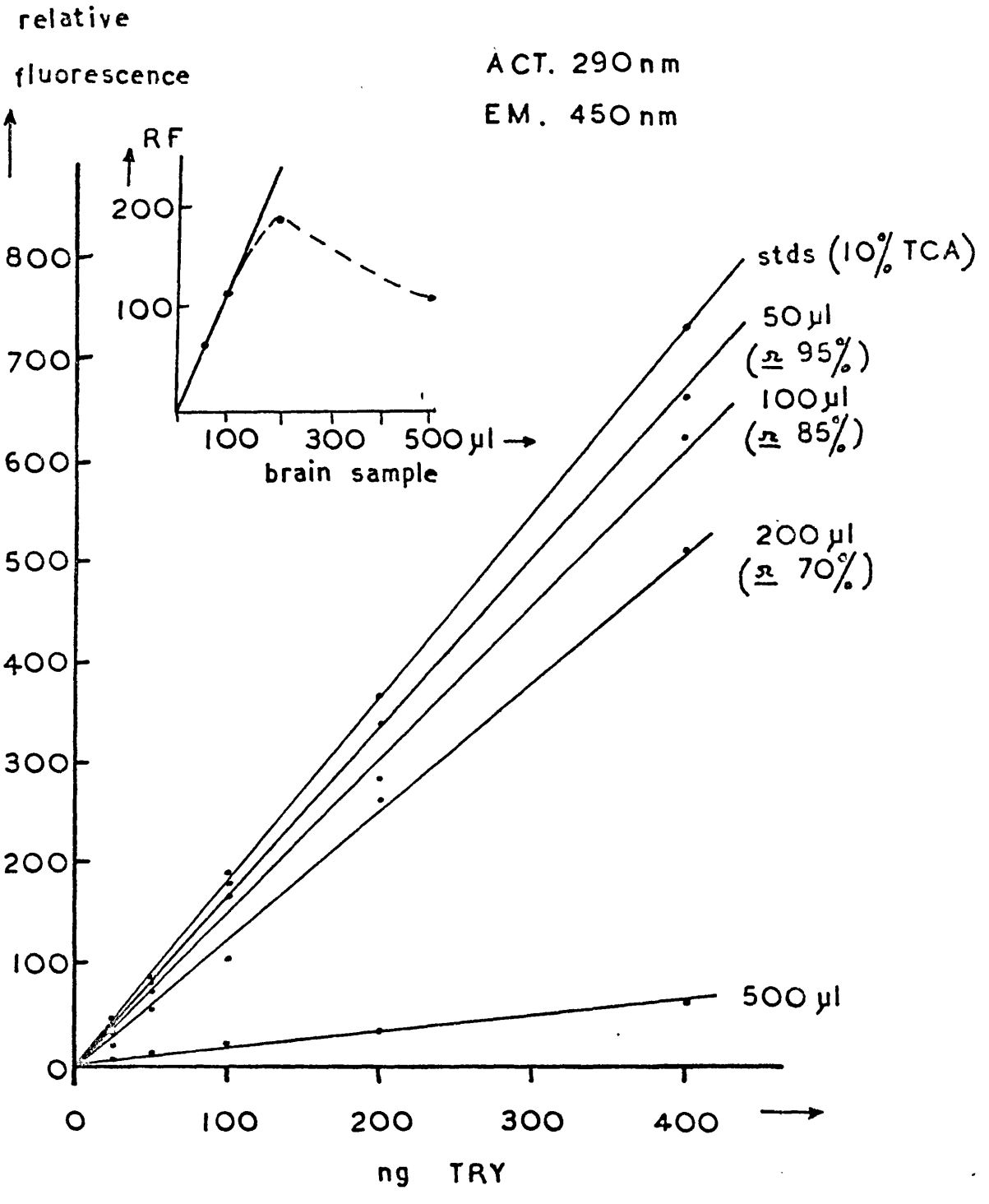


Figure 16 The relative fluorescence of TRY standards measured in the presence of increasing amounts (50, 100, 200 and 500µl) of a protein - precipitated brain extract.

Inset - the relative fluorescence of TRY measure in brain extract samples: emission 450nm, activation 290nm

and 1.8ml 10% TCA (w/v) containing 3 x 10^{-4}M $FeCl_3$ and read at excitation 290nm and emission 450nm. Fig. 14 shows that the relative fluorescence was proportional to increasing sample volumes up to 300µl.

Expt. 5 Standard curve for L-TRY in the presence of the ultrafiltrate used to determine 'free' TRY.
10µl of the following standard TRY solutions:- 25, 50, 100, 200 and 400µg/ml were added to 1ml of distilled water and 1ml of ultrafiltrate (for preparation refer to section 3.2.2). 100µl aliquots from each sample were made up to 1ml with distilled water and assayed as described in Expt. 4. Fig. 15 shows that no interference occurs.

Expt. 6 Standard curve for L-TRY in the presence of increasing amounts of a TCA proetin-precipitated brain extract.
The brain extract was prepared as described in section 3.2.1. 0-500µl extracts containing 25, 50, 100, 200 and 400ng of L-TRY were made up to 1ml with distilled water and assayed as described in Expt. 4. Fig. 16 shows the reduction in the relative fluorescence values for standards compared to a control curve as the volume of extract present in the mixture is increased although linearity is maintained. This experiment was then repeated using a 200µl extract but with 100ng norharman added to each sample before heating. As seen in Fig. 17 the values for norharman were unaffected indicating

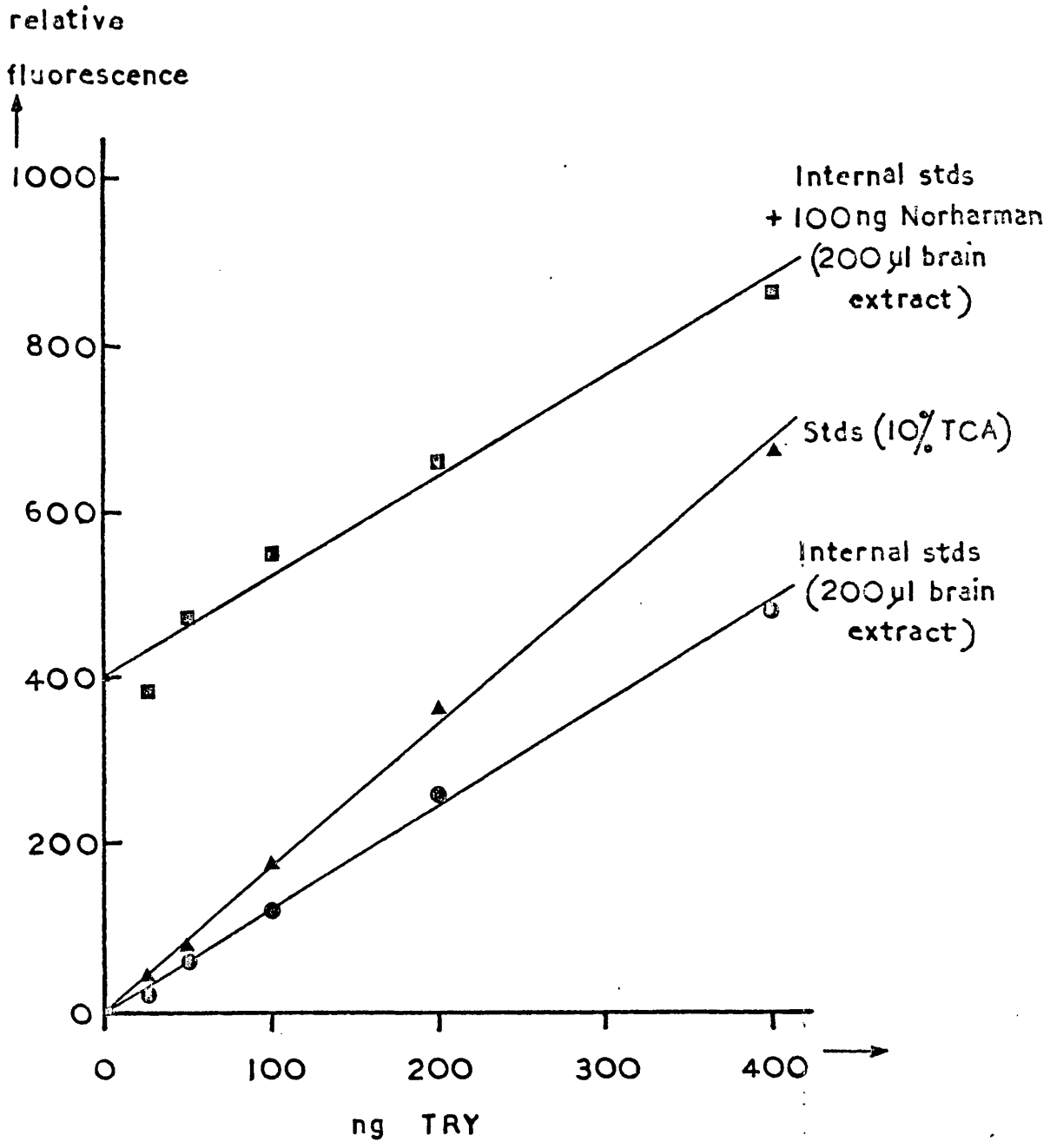


Figure 17 *The relative fluorescence of TRY standards measured in the presence of a) 200µl protein-precipitated brain extract b) 200µl brain extract + 100ng norharman: emission 450nm, activation 290nm*

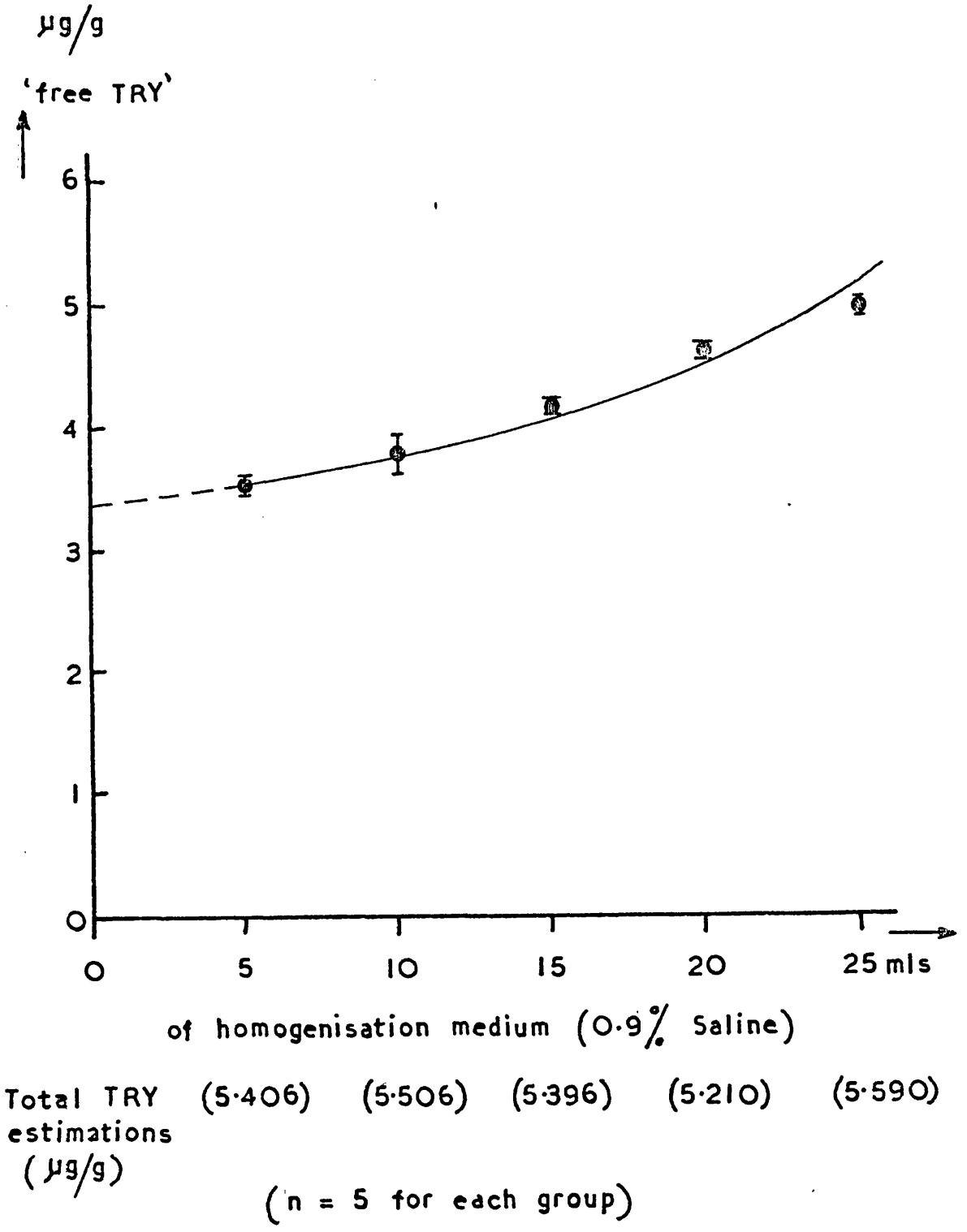


Figure 18 The effect of increasing the volume of homogenisation medium on the concentration of 'free' TRY in the rat brain. Total brain TRY estimations measured at each volume are expressed. (concentrations = µg/g wet brain weight)

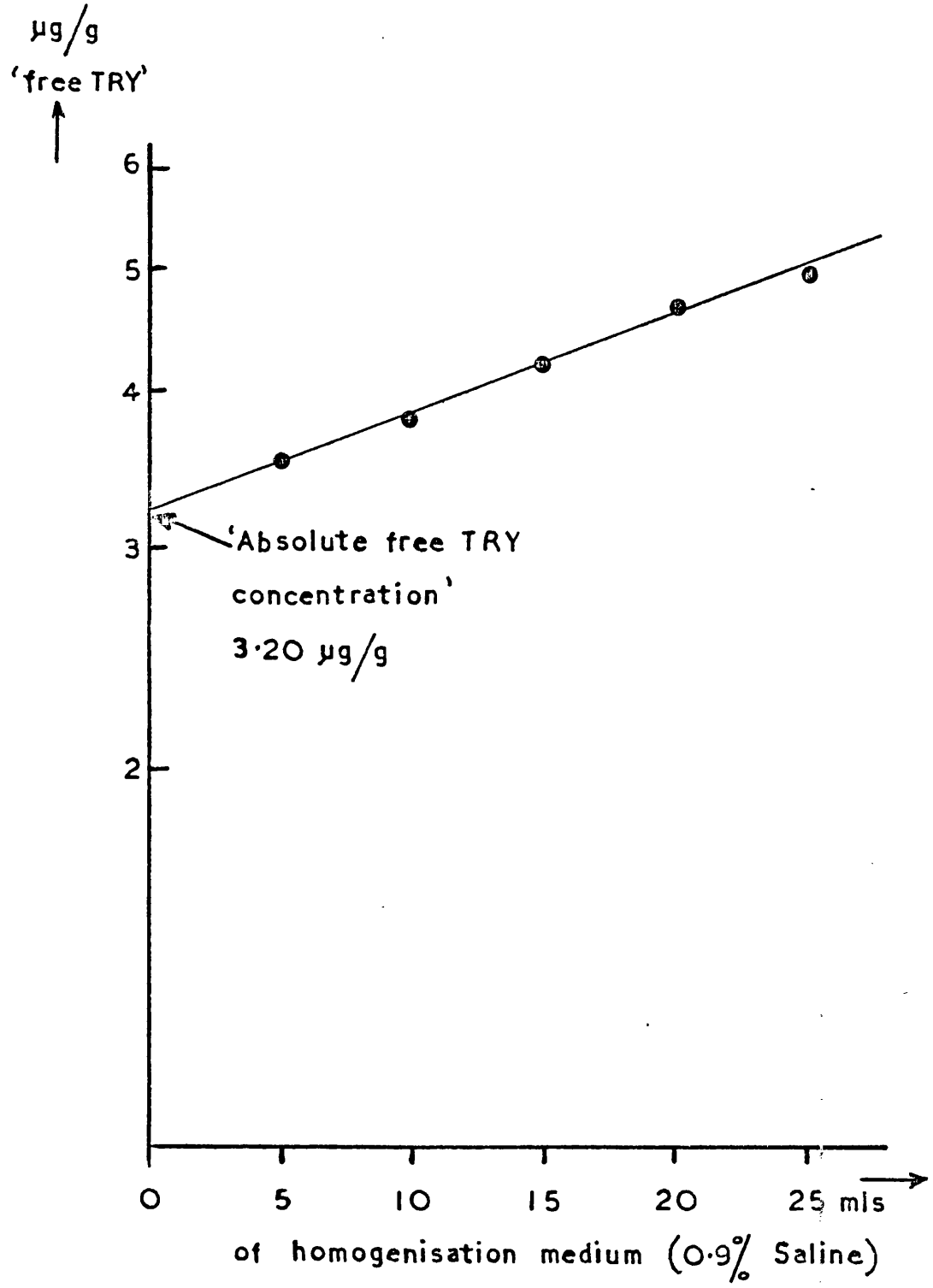


Figure 19 Data from the previous graph expressed on semi-log scale. Extrapolation to the y axis represents the 'absolute free TRY concentration' in the rate brain. Concentration = µg/g wet brain weight

the reduced fluorescence values for the standards prepared in the presence of brain extracts was not due to quenching of the final product.

Expt. 7 The determination of total, protein bound and 'free' TRY in the rat brain.

The assay was carried out as described in the method section 3.2. Fig. 18 shows the effect of increasing the volume of homogenisation medium on the amount of 'free' TRY in the rat brain. This data can be re-plotted by changing the y axis to a log scale and retaining the x axis as a linear scale. The values now give a straight graph (Fig.19) which on extrapolation to the y axis gives a value of 3.20 µg/g for the absolute concentration of 'free' TRY in the rat brain. Total TRY values are quoted on Fig. 18.

The 24-hour variation of total, bound and 'free' TRY in the rat brain

The assay was carried out as described in section 3.2.4. The results are shown in Fig. 20 and Table 1. Having calculated a value for the 'absolute free' TRY concentration in the brain (Expt. 7), the results obtained for 'free' TRY over 24 hours could thus be corrected to give 'absolute free' TRY values. As can be seen in Fig. 20 the circadian variation for 'absolute free' TRY levels closely follows that for total TRY.

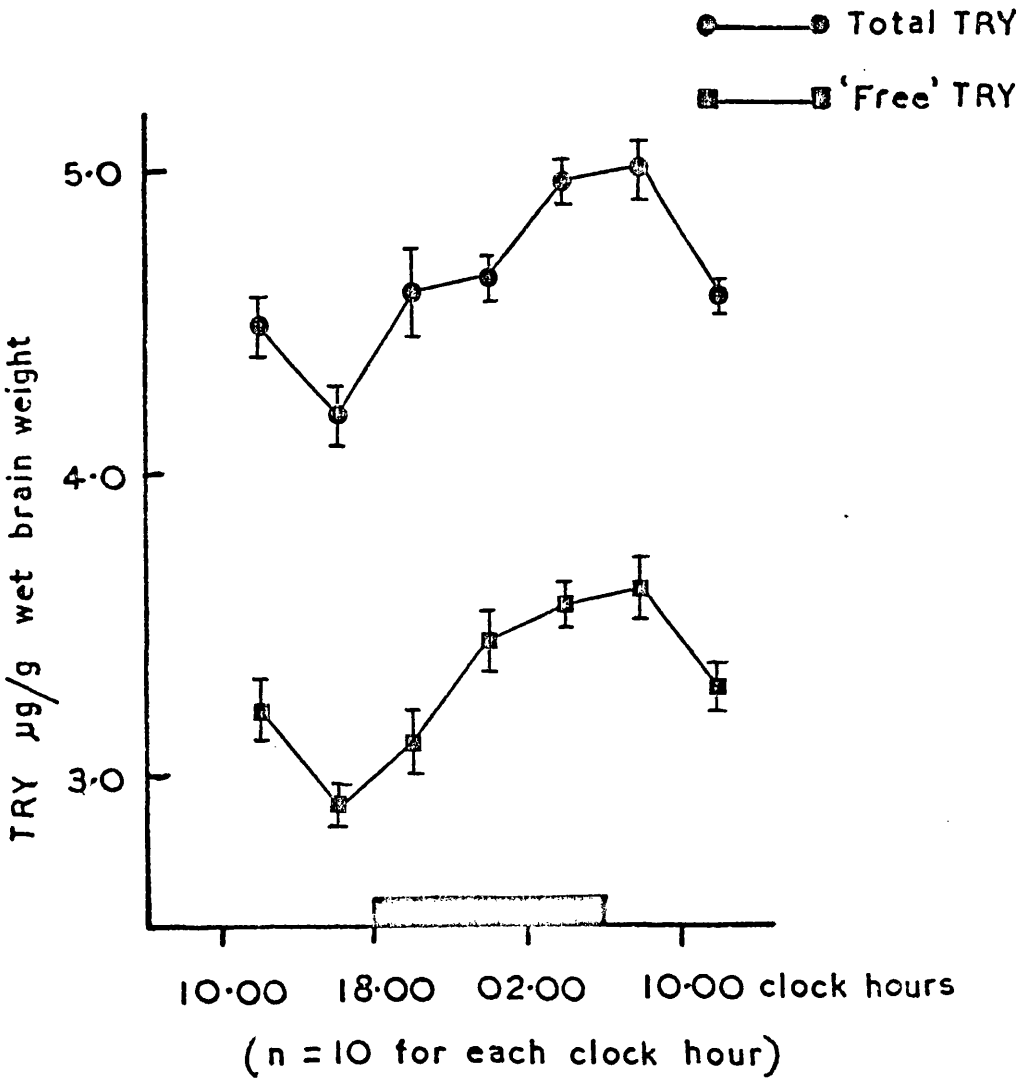


Figure 20 The 24-hour variation of 'free' and total TRY concentrations in the rat brain

Table 1 The 24-hour variation of total, protein bound and 'free' tryptophan in the rat brain.

	µgl/g			
Clock Hour	Total	'Free'	'Absolute Free'	Bound
12.00	4.508	4.209	3.215	1.293
16.00	4.224	3.908	2.914	1.310
20.00	4.626	4.119	3.125	1.501
24.00	4.706	4.462	3.468	1.238
04.00	4.943	4.589	3.595	1.348
08.00	5.063	4.621	3.627	1.436
12.00	4.603	4.221	3.283	1.320

n = 10 for each clock hour.

3.4 Discussion

Hopkins and Cole (1903) first prepared harman from TRY. Unknown to them at the time, acetaldehyde, as a contaminant, had condensed with TRY and the side chain had subsequently cyclised in the presence of $FeCl_3$ forming harman. Using the Pictet-Spengler reaction, Kermack et al., (1921) proved the correctness of the above sequence by synthesising norharman and harman with formaldehyde and acetaldehyde respectively. Hess and Udenfriend (1959) adapted this method for the measurement of tryptamine in tissues using hydrogen peroxide as the oxidising agent, no data however, was given as to the limits and optimal conditions of the method specifically applied to TRY determination. Denkla and Dewey (1967) produced the same yield from TRY (40% in 40 minutes) as had been reported by the previous authors for tryptamine. To improve the method, they used $FeCl_3$ as the oxidising agent instead of hydrogen peroxide and this increased the yield to 70% when heated at atmospheric pressure for 1 hour. Of the naturally occurring TRY derivatives tested, only tryptamine produced any appreciable fluorescence; one tenth of the molar fluorescence of TRY.

In this work, this simple, rapid and sensitive method was adapted to measure TRY in the brain. Perchloric acid could not be used as the denaturing agent in this assay because as seen from Fig. 10, it interferes with the assay so TCA

was used. Denkla and Dewey (1967) read their samples at excitation 370nm and emission 450nm. On scanning a 5µg sample of incubated tryptophan and 5µg non-incubated norhaman (in 0.1N HCl), the emission peak occurred at 450nm yet two excitation pecks were observed (Fig.11) at 290nm and 370nm, the former being greatest. The samples in this experiment were therefore read at excitation 290nm and emission 370nm.

Denkla and Dewey (1967) found that if norharman was added to pre-incubated reagents or incubated with the reagents, the fluorescence was 20% greater than if the former was added to non-incubated reagents. This increase in fluorescence appeared to be due to a change in the constituents of the reaction mixture during incubation and not to a change in the structure of norharman. Further investigation showed that $FeCl_3$ not formaldehyde or TCA quenched the fluorescence in a non-incubated sample. A decrease in $FeCl_3$ concentration during incubation accounted for the subsequent increase in fluorescence. Even after incubating some residual quenching by $FeCl_3$ remained. Further studies with the quenching caused by $FeCl_3$ revealed that the quenching was concentration-dependant and that it was completely reversed by dilution of $FeCl_3$, with water to a lower concentration. These observations were verified when the effect of 2.5×10^{-4}M $FeCl_3$ on the fluorescence of non-incubated norharman was investigated (Fig. 13). The

standard curve for norharman was reduced by approximately 20% when in the presence of $FeCl_3$. Increasing the volume of ultrafiltrate used to determine 'free' TRY produced a linear increase in fluorescence up to 300µl (Fig.14). No interference was observed when TRY standards were measured in 100µl ultrafiltrate samples (Fig.15). This was not the case however when standards were determined in the presence of increasing volumes of denatured brain extracts. As seen from Fig. 16 the recovery of TRY standards in 50, 100 and 200 µl extracts was 95%, 85% and 70% respectively, yet linearity was maintained. This effect was not due to quenching of the final product as 100ng norharman incubated with standards in 200 µl brain extracts gave the same fluorescence values as 100ng norharman samples incubated with reagents alone. 100 µl was selected for the routine assay since it gave an adequate recovery and was large enough to give accurate readings consistently.

The 'free' TRY measured in this experiment is defined as the non-protein bound fraction in the rat brain. The disadvantage of using a whole brain homogenate is that not only are levels in the neurons estimated but also those in glia and capillary vessels. There is no direct contact between neurons and their processes and the vascular system as the former are suspended within a sponge of neuroglial tissue (Glees, 1973). Glial tissue must therefore have a metabolic transport function, as well as a supportive role.

Also this 'free' TRY is not only available for amine synthesis in serotonergic neurons but is a precursor for melatonin in the pineal gland and for protein synthesis and turnover. Since about 10% of brain weight is protein and since each aromatic amino acid comprises about 5% of the total amino-acid content of the protein, about 5mg of each amino acid is incorporated into protein per gram of brain. In contrast, the amines NA and 5-HT are only 10^{-4} as abundant since they are found in concentrations of about 0.5 µg/g of brain. Even if one takes into account the more rapid turnover of amines, the proteins remain the major product derived from aromatic amino acids in the brain.

Ideally, one would like to measure the amount of this 'free' TRY which is available only to serotonergic neurons but this is not possible. As seen from Fig. 20 the circadian variation of 'free'TRY in the brain follows closely that for total TRY. This indicates that some other factor such as uptake of TRY into the neurons or the activity of the synthetic enzymes, tryptophan hydroxylase and/or 5-HTP decarboxylase could have a regulating function in order to explain the poor correlation between the circadian rhythm in brain TRY and 5-HT. Hery et al., (1972) showed an enhanced accumulation of {^{3}H} TRY in vivo in the light period during physiological or physical stimulation of 5-HT synthesis and suggested the implication of an active transport process to accelerate the transport of TRY into serotonergic neurons. The presence of a high affinity transport system was confirmed in synaptosomal

preparations (Grahame-Smith & Parfitt 1970; Knapp and Mandell,1972), it could be postulated that this system is a unique process of serotonergic neurons similar to the high affinity transport system of choline in cholinergic neurons (Kuhar et al.,1973). However, this is not the case as the systems studied have a wide specificity and also high affinity transport of TRY has been demonstrated in synaptosomal preparations free of serotonergic terminals (Kuhar et al.,1972) and in various cultured cells such as glial cells, fibroblasts and neuroblastoma clones (Bauman et al.,1974). For these reasons it appears unlikely that synthesis of 5-HT is controlled by a step regulating the transport of TRY across the synaptosomal membrane and thus determining a crucial intracellular concentration of TRY in the vicinity of tryptophan hydroxylase.

I therefore decided to look at the activity of 5-HTP decarboxylase and tryptophan hydroxylase over 24 hours. Preliminary findings in our laboratories (Hillier and Redfern,1976) had indicated the possible existence of a circadian rhythm for 5-HTP decarboxylase so it was decided to investigate this more closely.

CHAPTER FOUR

The activity of 5-HTP decarboxylase measured over 24 hours

4.1 Introduction

The enzymatic synthesis of the biogenic amines 5-HT, DA and NA involves decarboxylation of the corresponding amino acids, 5-HTP and DOPA. Dopa decarboxylase was the first enzyme in the catecholamine biosynthetic pathway to be discovered (Holtz et al., 1938). After the isolation and identification of 5-HT (Rapport et al., 1948; Rapport, 1949), Clark, Weissbach and Udenfriend (1954) found significant DOPA decarboxylase activity in their most highly purified preparations of 5-HTP decarboxylase from guinea-pig kidney. However the pH optima and cofactor requirements varied. In these early studies it was not possible to obtain separation of the two activities. Also the studies did not fully consider the fact that DOPA is a poor substrate to assay because it reacts with the coenzyme, pyridoxal phosphate to form a tetrahydroisoquinoline derivative (Schott and Clark, 1952). Yuwiler, Geller and Eiduson (1960) subsequently showed that there was competition between 5-HTP and DOPA for decarboxylation and that various inhibitors had similar actions on both decarboxylations. Using an enzyme preparation purified 50-100 fold from guinea-pig kidney, Lovenberg et al., (1962) concluded that not only DOPA and 5-HTP were decarboxylated by the same enzyme but that the specificity of the enzyme was sufficiently broad to utilize all the naturally occurring aromatic amino acids including histidine and certain α methyl derivatives. They also showed competitive substrate inhibition and inhibition of all the activities by α methylamino acids. For these reasons, they proposed that the enzyme be named 'aromatic L-amino acid

decarboxylase'. The close similarity in regional distribution of DOPA decarboxylase and 5-HTP decarboxylase activities in feline brain (Kuntzmann et al., 1961) provided experimental support for extrapolating the characteristics of the renal enzyme, particularly its broad substrate specificity, to the corresponding reactions in the brain. The possibility of more than one decarboxylase for aromatic amino acids in different organ systems was raised however when a partially purified DOPA decarboxylase preparation from liver was reported to exhibit significant differences in pH optima, substrate specificity and cofactor requirements from those of the kidney decarboxylase (Awapara et al., 1962).

All the above investigations, were limited because highly purified enzyme preparations were not available. Christenson et al., (1970) obtained preparations of hog kidney decarboxylase which were 99% pure as indicated by disc gel electrophoresis, sedimentation and immunological techniques. The purified enzyme had a molecular weight of 112,000 daltons and was associated with tightly bound pyridoxal-5-phosphate (P5P). The enzyme decarboxylated DOPA, 5-HTP, TRY, phenylalanine and tyrosine at readily measurable rates. Slow decarboxylation of histidine also occurred. A subsequent report (Christenson et al., 1971) showed that the anti-serum to this purified hog kidney enzyme also reacted with preparations from other tissues and other species suggesting that the same enzyme or a close homologue was widely distributed. Anti-serum titrations have shown that both DOPA and 5-HTP decarboxylase activities were lost at the same rate (Christenson et al., 1972) again

suggesting that a single enzyme is responsible for both activities. Further evidence to support the findings that the enzyme has the ability to utilise both DOPA and 5-HTP was shown when large loading doses of 5-HTP increased indoleamine fluorescence in catecholamine containing neurons as well as serotonergic neurons (Butcher et al., 1972). In contrast, loading doses of L-DOPA increased catecholamine fluorescence in both serotonergic and dopaminergic neurons (Butcher et al., 1970).

This evidence must be reconciled however with the observations of Sims and Bloom (1971), who undertook a detailed study of DOPA decarboxylase and 5-HTP decarboxylase to establish assay characteristics and distribution in the rat brain by using a sensitive microradiometric technique. The two activities exhibited widely different optima for pH, temperature and substrate concentrations. The activity of 5-HTP decarboxylase was stimulated 2-fold by added P5P and was relatively resistant to antagonists of the latter. By contrast, the activity of DOPA decarboxylase was stimulated 20-fold by added coenzyme and could be completely inhibited by carboxyl-trapping agents. Dopa decarboxylase activity in subcellular fractions of brain was associated predominently with the soluble fractions and its distribution in the various fractions closely paralled that of lactic acid dehydrogenase. These results are in agreement with previous subcellular fractionation studies on renal and cerebral DOPA decarboxylases (Laduran and Belpaire, 1968; McGear et al., 1965). A

cytoplasmic location for DOPA decarboxylase is also consistent with the localisation of its product DA (Laverty et al.,1963) in supernatant fractions. 5-HTP decarboxylase activity in brain was distributed almost equally between soluble and particulate fractions and its distribution within the particulate fractions differed from that of succinic acid dehydrogenase. These findings agree with earlier experiments on the subcellular localisation of cerebral 5-HTP decarboxylase activity (Rodriguez De Lores Arnaiz and DeRobertis, 1964). Similarly, the regional distributions of the two decarboxylases in rat brain did not parallel one another. Rostal areas exhibited the highest DOPA decarboxylase activity e.g. the activity in the corpus striatum was 11-fold greater than in the whole cerebellum. The activity of 5-HTP decarboxylase relative to that of DOPA decarboxylase was greater in hindbrain regions (medulla-pons and cerebellum), although its maximal activity per unit weight was in the telencephalon. It was suggested that the enzymes might be so closely related structurally as to be identical by the immunological criteria used by Christenson et al., (1972).

Bender and Coulson (1972) had also found that electrophoretic studies on rat liver DOPA and 5-HTP decarboxylase consistently failed to show any separation of the two activities under a variety of conditions indicating that a single protein is responsible for both activities (Coulson et al., 1969; Bender and Coulson, 1973). Yet they demonstrated that the two enzymes have different pH optima for decarboxylation (DOPA at pH 6.6 - 7.0 and 5-HTP pH 7.8 - 8.0) but are mutually competitive.

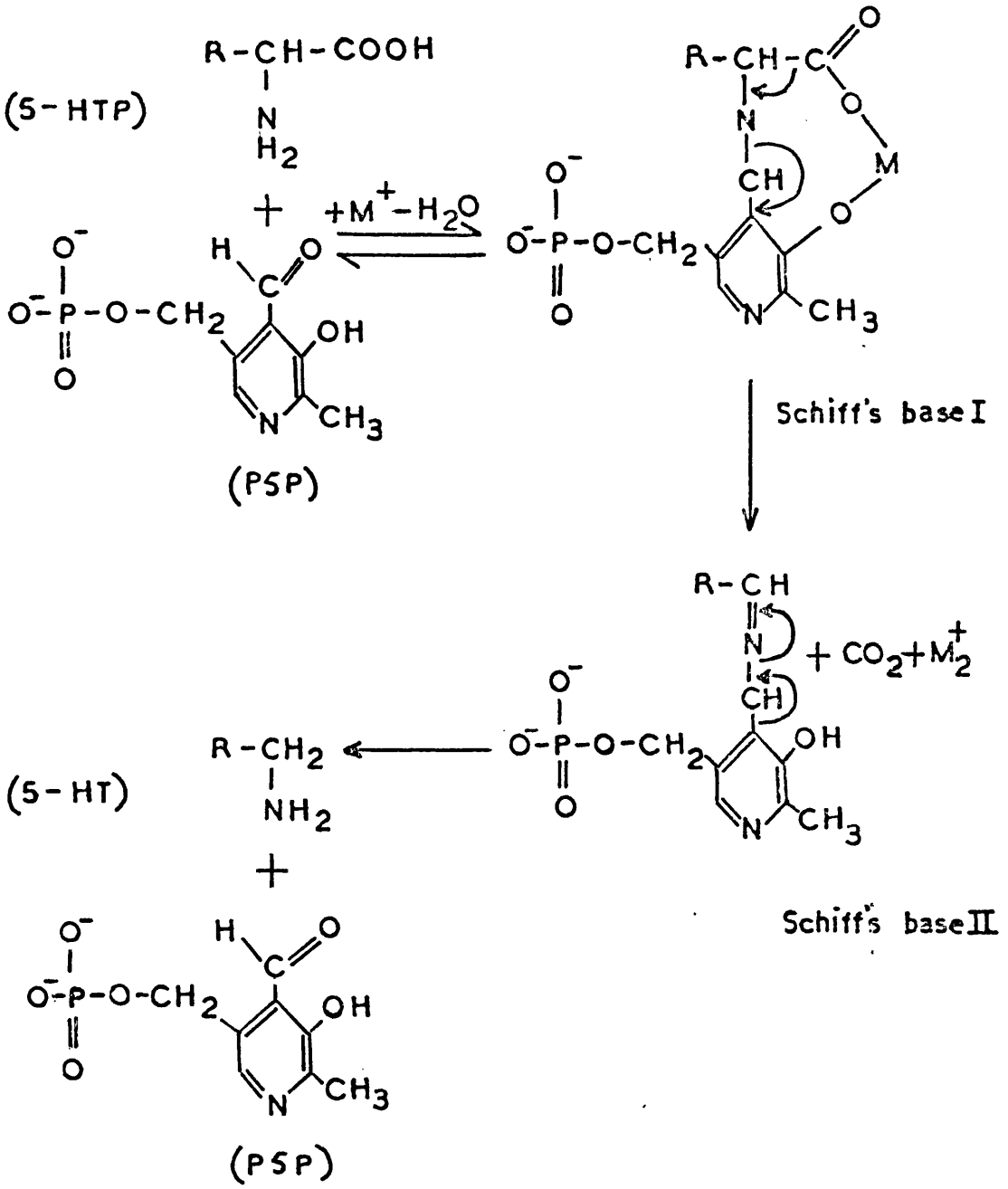
They also showed that the ratio of activity of the liver enzyme towards the two substrates could be altered by mild treatments, such as might be used in solubilization of brain preparations. Dopa decarboxylase activity was preferentically inactivated by sodium dodecyl sulphate treatment and 5-HTP decarboxylase by urea. Inhibition of DOPA decarboxylase by the detergent also relieved the inhibition of 5-HTP decarboxylation by DOPA and the corresponding observation applied for DOPA decarboxylation inhibited by 5-HTP. In an attempt to explain their results, Bender and Coulson (1972) proposed that since there was only one molecule of P5P per molecule of enzyme (Christenson et al., 1970) it must be assumed that there can only be a single catalytic site on the molecule but the difference in pH optima for the two substrates might reflect two separate affinity sites which could be differentially inactivated. This could explain the observations of Sims and Bloom (1971) on the brain enzyme, which being partially membrane bound or enclosed could be modified by the extraction techniques. Differences in the ionization of the affinity sites might then determine the pH optima towards the substrates and the differential effects of sodium dodecyl sulphate and urea could be explained by differential sensitivities of the two sites.

The existence of a single enzyme catalyzing the same type of reaction in different biosynthetic pathways is an unusual circumstance in mammalian biochemistry. The situation concerning brain tissue has not been clarified fully and I

believe the practise of using 5-HTP or DOPA interchangeably as substrates to assay 'aromatic L-amino acid decarboxylase' may provide misleading information in studies utilising brain homogenates. For this reason, 5-HTP has been used as the substrate to measure the activity of 5-HTP decarboxylase over 24 hours in this thesis.

Fig. 21 Mechanism of action of the co-factor pyridoxal-5-phosphate in the decarboxylation of 5-HTP.

The αamino group of 5-HTP reacts reversibly with the aldehyde group of P5P to form a Schiff's base.



M^+ is an electron-withdrawing structure contributed by the enzyme.

4.2 Method

4.2.1 Incubation

Male CFY rats (180-220g) were killed by decapitation, the brains dissected out and the pineal glands discarded. After weighing, each brain was homogenised in 8ml of ice-cold 0.32M sucrose using a teflon pestle and a glass tube homogeniser with a 0.01" radial clearance and revolving at 800 rpm. The homogenates were centrifuged at 1000g for 10 mins at $4^{o}C$, the supernatants collected and diluted (1vol + 3vols 0.32M sucrose) and 1ml of this preparation was made up to 2.2ml to form the final incubation mixture. This contained 0.45×10^{-5}M pargyline and 1.82×10^{-5}M P5P adjusted to pH 8.0 with phosphate buffer. Fig. 21 shows the mechanism of action of P5P in the decarboxylation of 5-HTP. The mixture was pre-incubated at $37^{o}C$ for 10 mins before the addition of 1.25×10^{-5}M DL-5-HTP*. The incubation was allowed to proceed for 15 mins and was stopped with 0.1ml 4N sulphuric acid. The mixture was centrifuged at 1000g for 10 mins to remove precipitated proteins.

4.2.2 Extraction

The 5-HT contained in the resulting supernatant was extracted and assayed by adapting the method described by Snyder et al., (1965).

*These were the optimal conditions under which the enzyme activity was measured. DL-5-HTP substrate concentrations sometimes varied however depending on the experiment performed and the appropriate value is quoted in the results.

Fig. 22 Incubation Method used for the kinetic studies

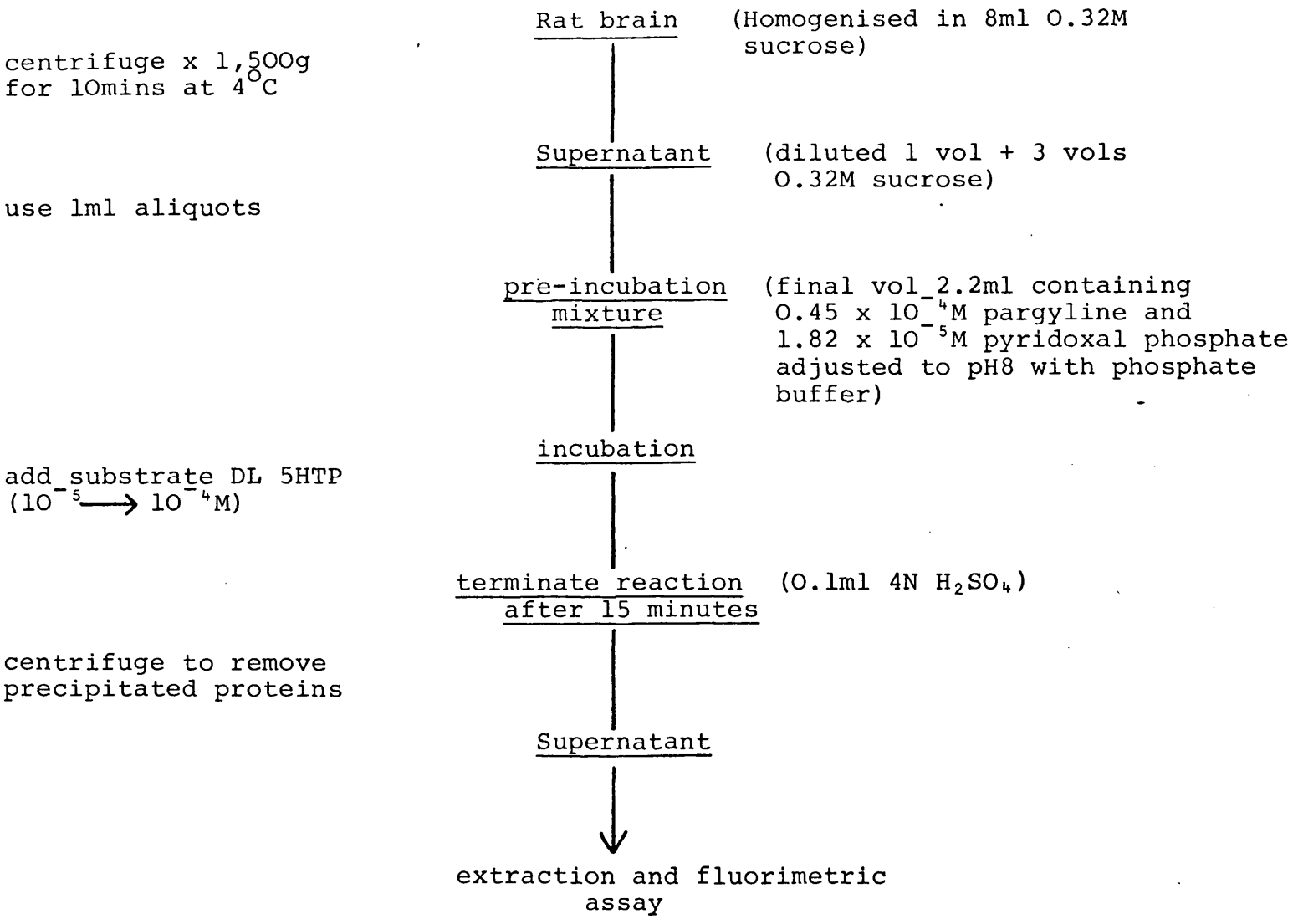
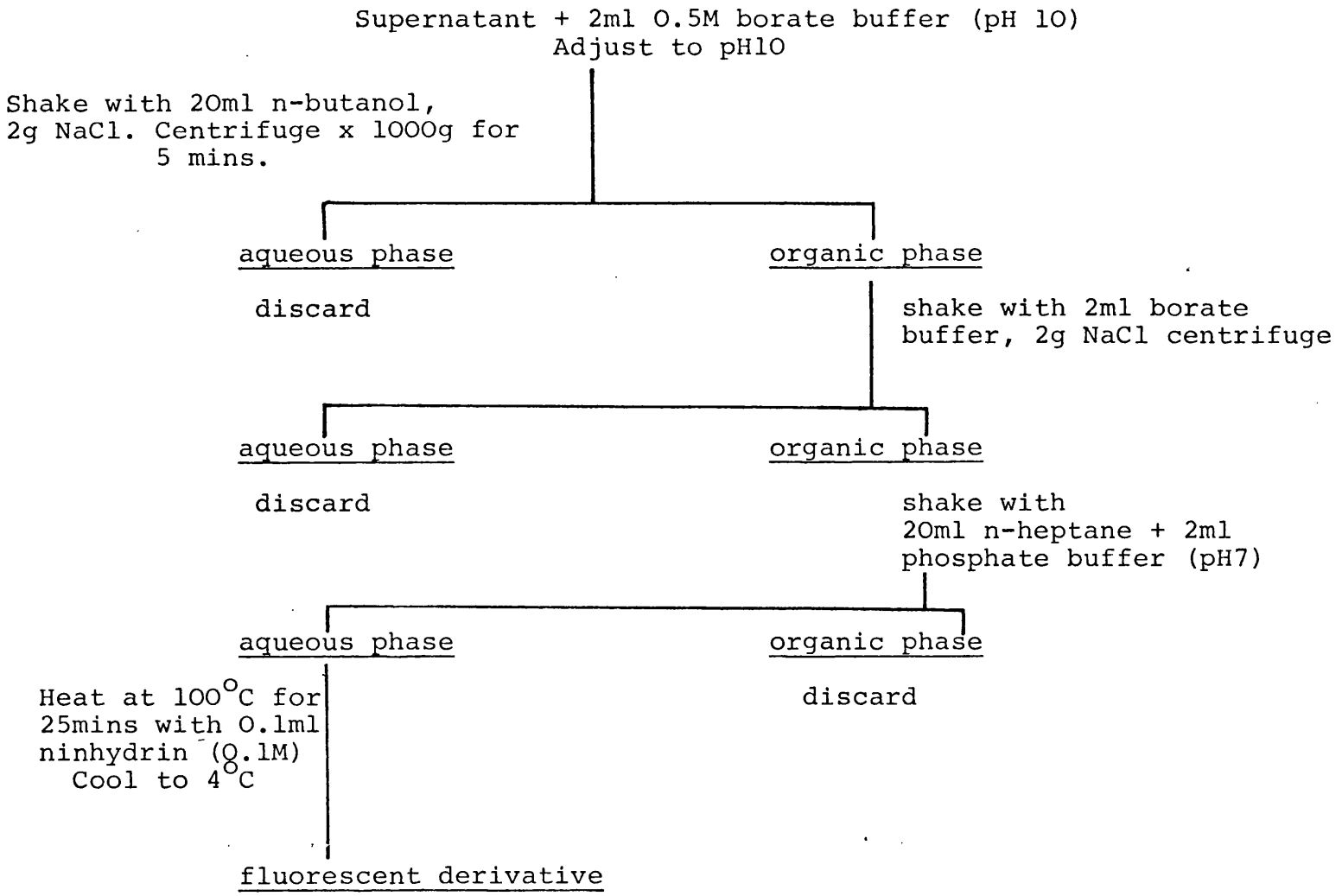


Fig.23 Extraction and assay method for 5HT.



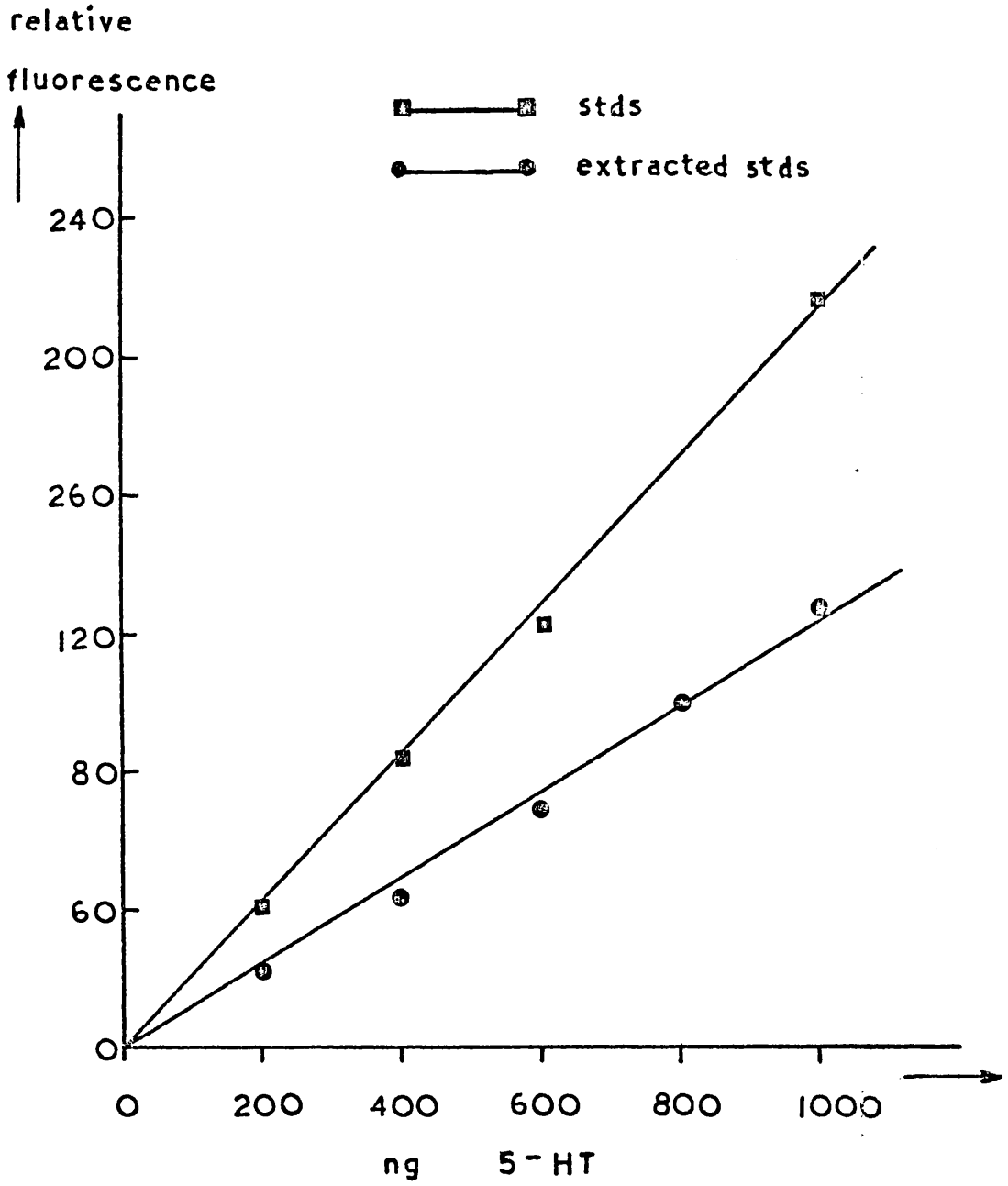


Figure 24 The relative fluorescence of 5-HT measured before and after organic extraction: emission 490nm, activation 385nm

Each supernatant was added to 2ml 0.5M borate buffer (pH10, saturated with NaCl and washed n-butanol). The pH was adjusted to pH 10 with 5N NaOH and this solution added to 20ml of washed n-butanol and 2g NaCl in a 125ml 'medical flat' bottle, which was shaken for 5 min and centrifuged at 1,000g for 5 min. The aqueous layer was discarded and another 2ml of borate buffer and 2g NaCl were added. The contents of the bottle were shaken and centrifuged at 1,000g for 5 min. 15ml of the n-butanol phase was transferred to another bottle containing 20ml n-heptane and 2ml of 0.05M phosphate buffer (pH7). This was shaken and centrifuged at 1,000g for 10 min. 1.2ml of the aqueous layer was added to 0.1ml of 0.1M ninhydrin, heated for 25 min at 100^{o}C and allowed to cool for 1 hour. The fluorescence was read in an Aminco Bowman spectrophotofluorimeter at activation 385nm, emission 490nm. Internal standards (500ng 5-HT) were added to the supernatant from the incubation, external standards (500ng 5-HT) were prepared in 0.32M sucrose and extracted as described. Figs. 22 and 23 illustrate a summary of the incubation and extraction methods.

4.3 Results

Expt. 1 A comparison of the fluorescence intensity of the ninhydrin derivative of standard and extracted 5-HT.

A curve of the relative fluorescence of extracted 5-HT standards over the range 50-1000ng was constructed and compared to that obtained for non-extracted standards covering the same range. Fig. 24 shows the fluorescent values produced

Fig. 25 The excitation spectra of 5-HT and extracted 5-HT.

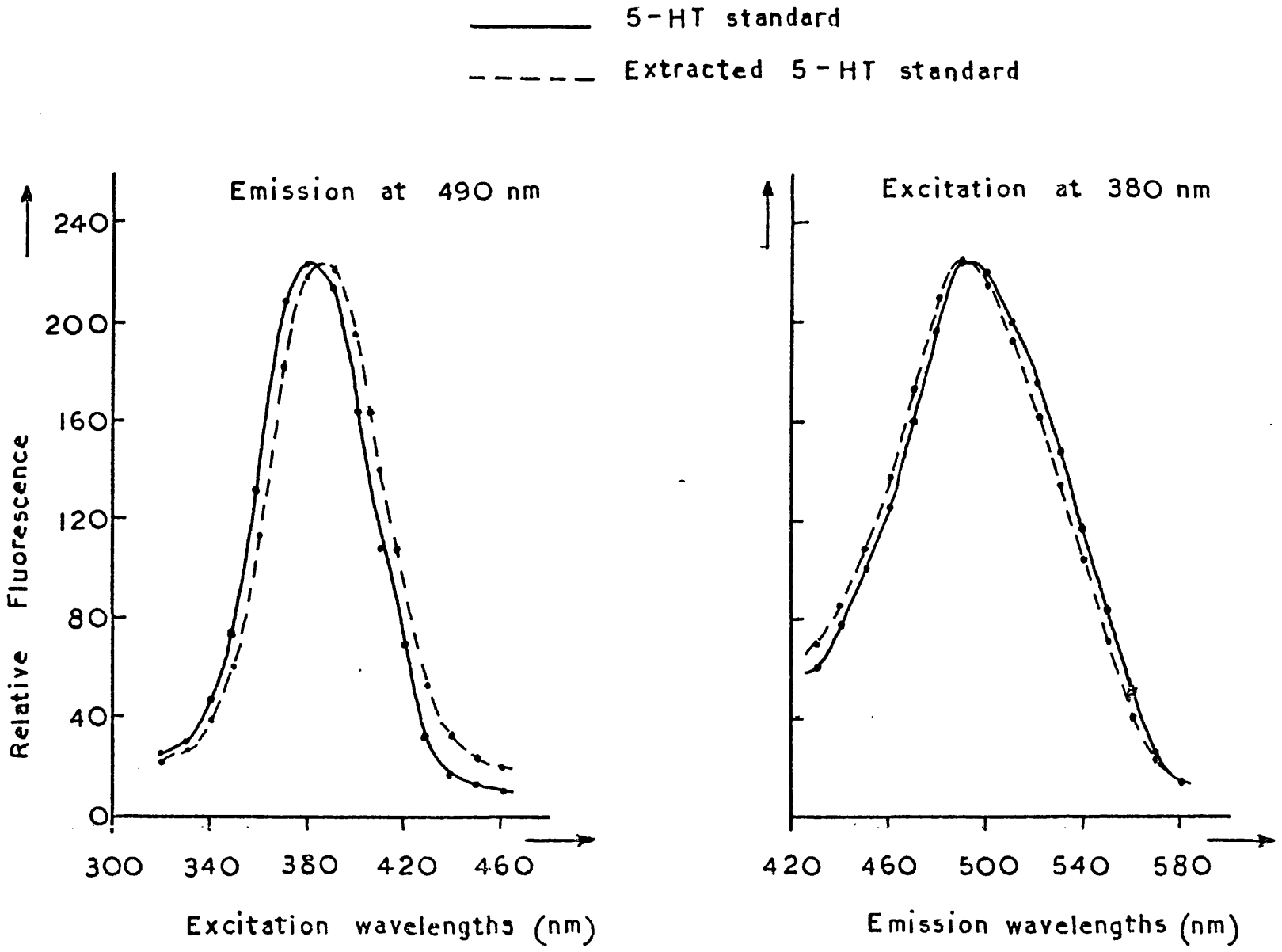
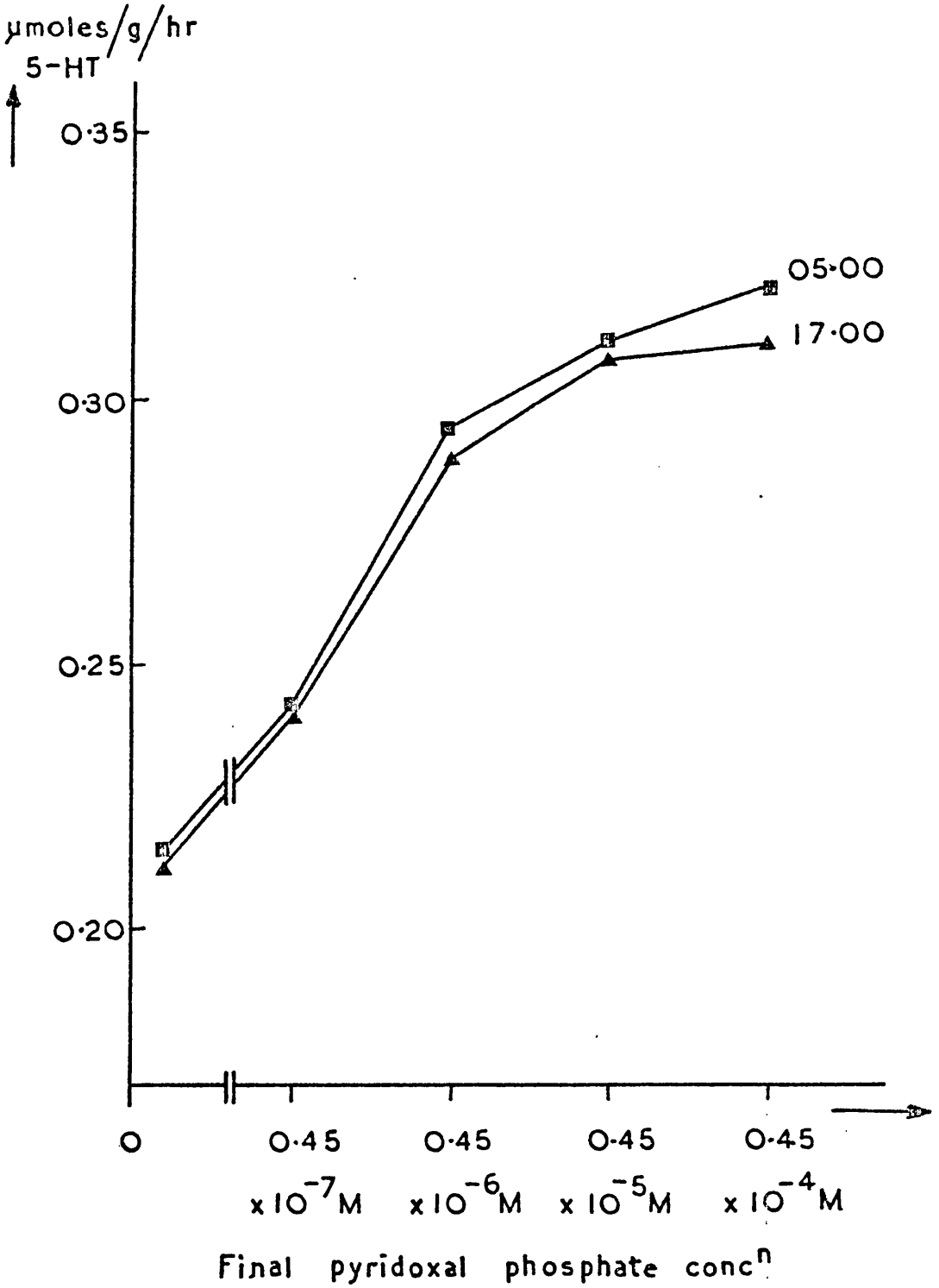

5-HT standard
Extracted 5-HT standard
Emission at 490 nm
Relative Fluorescence
240
200
160
120
80
40
0
300
340
380
420
460
Excitation wavelengths (nm)
Excitation at 380 nm
420
460
500
540
580
Emission wavelengths (nm)

Fig. 25



igure 26 *The effect of altering P5P concentration on 5-HTP decarboxylase activity. Velocity expressed as µmoles 5-HT formed g wet brain weight/hour. Conditions of incubation - pH8, 37°C, 15 min; pargyline 0.45 x 10^{-5}M, DL 5-HTP 1.25 x 10^{-5}M (final concentrations)*

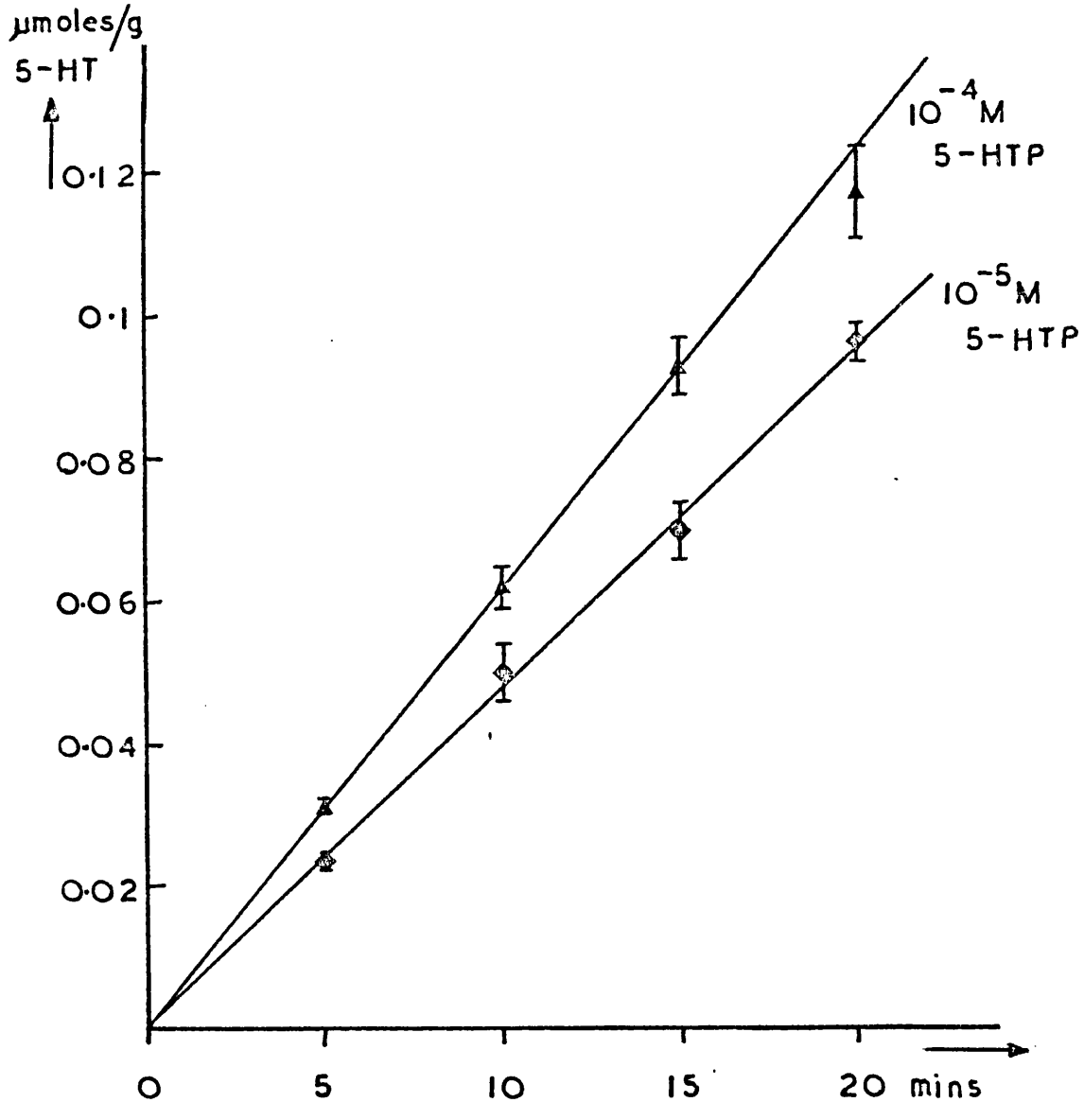


Figure 27 *The effect of increasing incubation time on 5-HTP decarboxylase activity at the highest and lowest concentrations of 5-HTP used for the kinetic studies.*

Velocity expressed as µmoles 5-HT formed/g wet brain weight/hour

Conditions of incubation - pH8, 37°C, 15 min;

pargyline 0.45 x 10^{-5}M, P5P 1.82 x 10^{-5}M (final concentrations)

a linear graph in both cases over the range chosen and the calculated recovery estimated as 56 ± 1.1%. Fig. 25 shows a fluorescence spectra of the ninhydrin derivatives of a standard and extracted 5-HT sample.

Expt. 2 The effect of altering P5P concentration on 5-HTP decarboxylase activity.

The effect of P5P on enzyme activity was assessed using preparations from rats killed at 05.00 and 17.50 hours. As seen in Fig. 26 the velocity of the reaction increased with increasing P5P concentrations until a maximum velocity was attained at 0.45×10^{-5} M P5P (final concentration). This effect was seen at both clock hours measured.

Expt. 3 The effect of increasing incubation time on enzyme activity at the highest and lowest substrate concentrations used for the kinetic studies.

Fig. 27 shows that at substrate concentrations of 10^{-4}M and 10^{-5}M the velocity of the reaction is linear over the time course measured.

Expt. 4 The estimation of Vmax and Km for 5-HTP decarboxylase at 17.00 and 05.00 hours.

The method used was that described in section 4.2 except a range of DL-5-HTP concentrations :- 10^{-5}M, 1.25×10^{-5}M, 2×10^{-5}M, 4×10^{-5}M, 10^{-4}M (final concentrations) was used. The results were plotted as velocity against substrate (Fig.28)

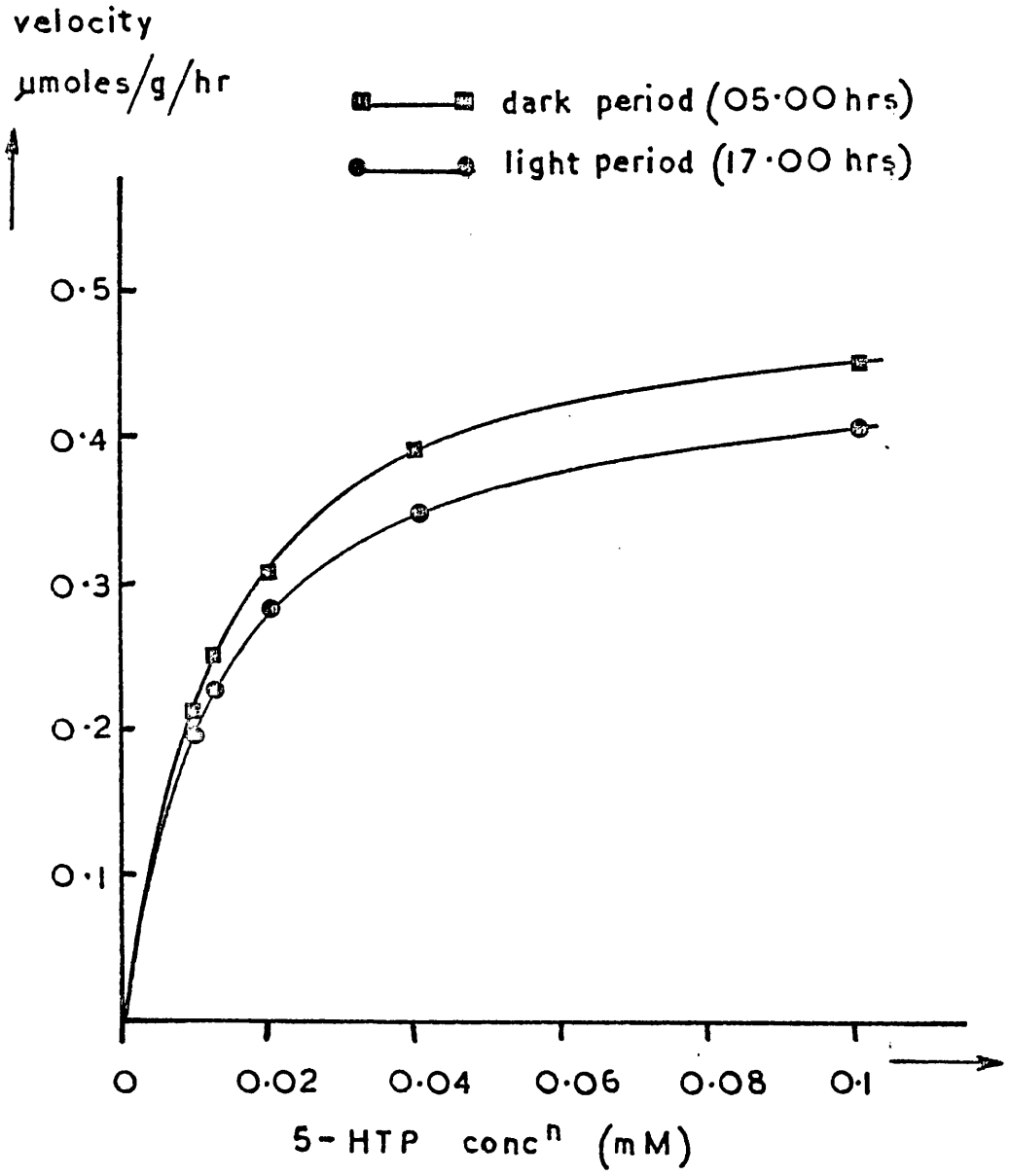


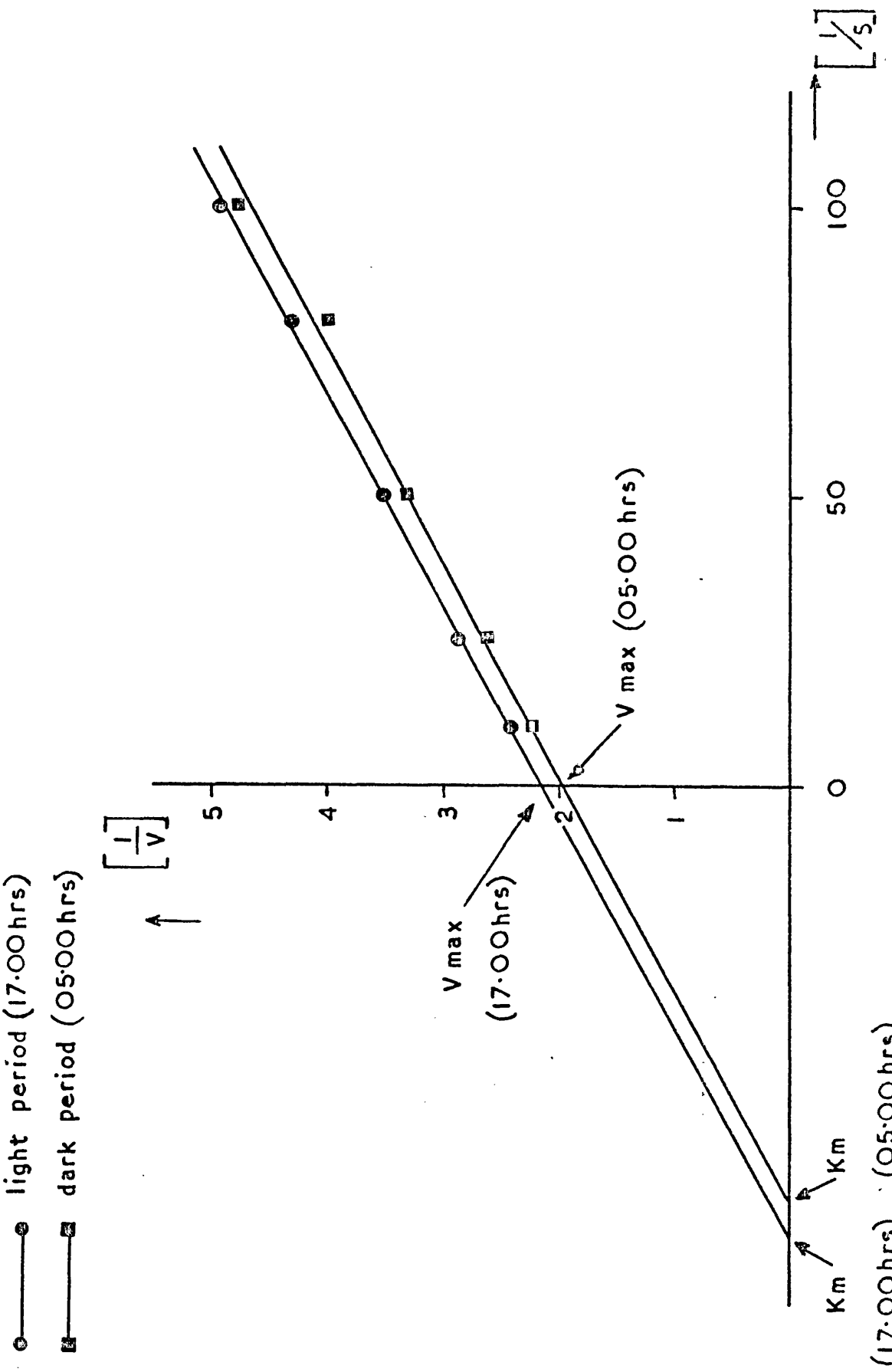
Figure 28 *The activity of 5-HTP decarboxylase as a function of substrate concentration.*

Velocity expressed as µmoles 5-HT formed/g wet brain weight/hour

Conditions of incubation - pH8, 37°C, 15 min;

pargyline 0.45 x 10^{-5}M, P5P .82 x 10^{-5}M (final concentrations)

Fig. 29 Data from the previous graph expressed in the form of a Lineweaver - Burk plot.
V = velocity (μmoles 5-HT formed/g wet brain weight/ hour.
S = substrate concentration (mM).


light period (17·00hrs)
dark period (05·00hrs)
1/V
5
4
3
2
1
0
50
100
1/S
V max (05·00 hrs)
V max (17·00hrs)
Km (17·00hrs)
Km (05·00hrs)

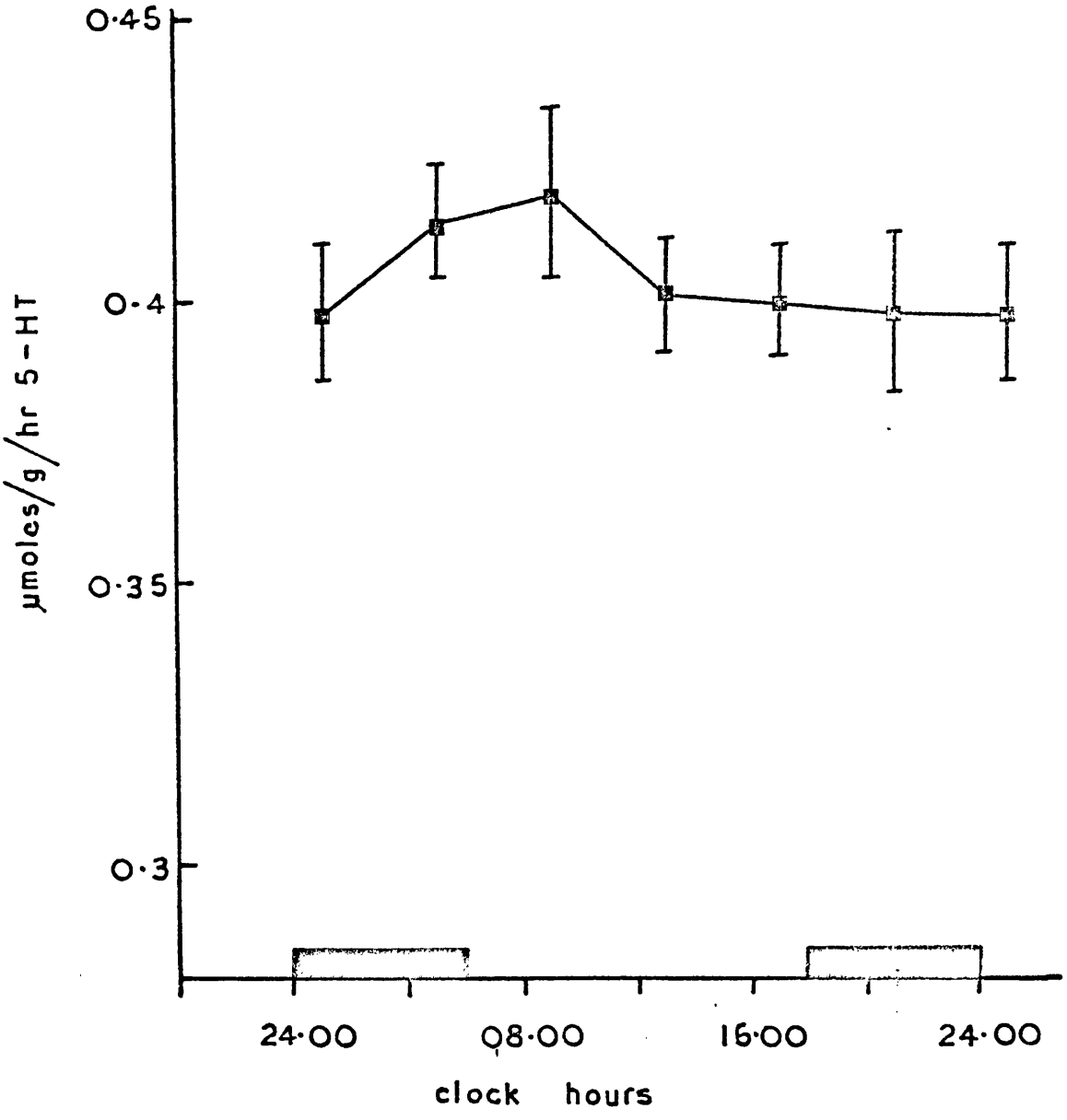


Figure 30 *The 24 hour variation in 5-HTP decarboxylase activity in the rat brain.*

Velocity expressed as µmoles 5-HT formed/g wet brain weight/hour

Conditions of incubation - pH8, 37°C, 15 min;

pargyline 0.45 x 10^{-5}M, P5P 1.82 x 10^{-5}M,

DL 5-HTP 5 x 10^{-5}M (final concentrations)

n = 10 at each clock hour

and the reciprocal values used to construct a Lineweaver-Burk graph (Fig. 29). The Vmax and Km values for the two clock hours were as follows :-

Clock hour		Vmax	Km
(dark)	05.00	0.510 μmol $g^{-1}hr^{-1}$	1.39 x 10^{-5}M
(light)	17.00	0.465 μmol $g^{-1}hr^{-1}$	1.28 x 10^{-5}M

The activity of 5-HTP decarboxylase estimated over 24 hours.

In this experiment, the enzyme activity was measured at 4 hourly intervals over 24 hours and at one substrate concentration only 5 x 10^{-5}M (final concentration) as seen in Fig. 30, although a small variation in activity occurred between values estimated at 05.00 and 17.00 hours, the peak activity was reached at 09.00. The observed data were analysed by fitting the results to a sine curve, a geometric model with a natural, recognised rhythm. It is assumed that if the data do not significantly differ from the calculated sine curve, then a rhythm is in evidence, whose highest values do differ from the lowest values. The best fit sine curve has been calculated using the Fourier analysis and the experimental data compared to the calculated data by means of the x^2 test. The results were significantly different from the calculated sine curve indicating that no circadian variation existed.

4.4 Discussion

Following the observations of Jepson and Stevens (1953) that 5-HT developed strong fluorescent properties after exposure to ninhydrin and heat, Vanable (1963) developed a quantitative

Clock hour	μmol hr^{-1} g wet brain $wt.^{-1}$
01.00	0.398 $\pm$ 0.012
05.00	0.414 $\pm$ 0.010
09.00	0.419 $\pm$ 0.015
13.00	0.401 $\pm$ 0.012
17.00	0.400 $\pm$ 0.010
21.00	0.398 $\pm$ 0.014

Table 2 24-hour variation in 5-HTP decarboxylase activity in the rat brain.

fluorescence assay for 5-HT using standard solutions. The fluorescence was eight times more intense than the native fluorescence of 5-HT in strong acid solution. This technique was later applied to tissue samples and the specificity of the reaction was determined by Snyder et al., (1965). The peak fluorescence of the 5-HT ninhydrin product is achieved at pH 6.5, therefore 5-HT can be extracted into phosphate buffer and the ninhydrin reaction performed directly. It is necessary to leave the reacted samples for 1 hour as the fluorescence of the product increases approximately 10% during the first 20 minutes following incubation and then remains stable for 6 hours. A linear range of relative fluorescence occurs over 0.005 - 0.5 μg 5-HT/ml. A wide variety of related hydroxyindole and catechol compounds react with ninhydrin but only bufotenin and 5-HTP were reported to yield an appreciable amount of fluorescence (2% and 1% respectively).

In these expereiments the recovery of extracted 5-HT formed during incubation was 56 $\pm$ 1.1%. For the kinetic studies the range of 10^{-5}M to 10^{-4}M DL-5-HTP was chosen and the velocity of the reaction at these two substrate concentrations was measured over 20 mins to ensure linearity (Fig. 27). Most reaction curves are straight lines up to approximately 20% of the reaction and as long as measurements are made in this linear range, the rate of substrate utilisation is a true measure of enzyme activity.

Preliminary findings from our laboratories (Hillier and

Redfern, 1976) had indicated a circadian rhythm in 5-HTP decarboxylase the peak value occuring at 17.00 hour (i.e. 11th hour of dark), thus for the kinetic studies, this was the clock hour chosen at which the enzyme activity was measured as well as at 05.00 hour (i.e. 12 hour later). Initially, the effect of increasing P5P concentrations was measured at these two clock hours to establish whether the added cofactor was affecting activity differently. As seen from Fig. 26 this was not the case and activity increased to a maximum at a P5P concentration of 0.45×10^{-5}M at both clock hours.

The experimental data from the kinetic studies were displayed in the traditional form of Lineweaver-Burk plots for the two clock hours and were analysed statistically by fitting rectangular hyperbolae using a maximum likelihood computer program (MLP, Ross et al.,). The difference between the two lines was small but the values at 17.00 hour were always greater than 05.00 hour. The statistical analysis showed a highly significant separation between the lines but no significant difference between their slopes. However, when the activity of the enzyme was measured over 24 hours, (Fig. 30) although a difference still existed between 17.00 and 05.00 hours, no statistically significant circadian variation was observed.

It is difficult to explain the functional significance of this observed difference for the rate of 5-HT synthesis at 2

clock hours when apparently no 24-hour variation exists. Also the latter does not substantiate the preliminary findings in our laboratories. This could be explained possibly by animal strain differences (the Sprague-Dawley strain compared to CFY) or method differences. In this experiment a fluorimetric method was used which could be considered less sensitive than as isotopic method used originally. However, the isotopic method required a far lengthier extraction procedure to separate the radioactive substrate from product causing a low recovery and a blank value which was still high. With the fluorimetric method, the extraction procedure was more simple as any increased 5-HTP present in the final solution to be heated with ninhydrin did not interfer because it yielded negligible amounts of fluorescence. Thus recovery values were high and blank values low. Any increase in the degree of sensitivity gained from using an isotopic method was lost due to the extraction procedure necessary.

In attempting to explain the difference, perhaps the most relevant findings from earlier work were that when a purified enzyme extract was used, prepared by the method of Clark et al., (1954), no 24-hour variation was seen. Therefore, it was concluded that in the crude extract, the variation in the activity of the enzyme was due to other factors. There was no variation due to endogenous or exogenous P5P. Equally the availably of the precursor, did not appear to affect the activity of the enzyme since pretreatment with PCPA at different clock hours did not significantly alter the rates

of synthesis. It was suggested that as 5-HTP decaboxylase has the ability to utilize DOPA as a substrate perhaps competition between 5-HTP and DOPA could be a controlling factor.

Possibly, method and animal variation mask what could be consistently small fluctuations in enzyme activity or maybe the importance of endogenous 5-HTP levels should be considered. 5-HTP levels in the rat brain are low (Linquist et al., 1975), because the maximum velocity of tryptophan hydroxylase, the enzyme which converts TRY to 5-HTP is lower than that of 5-HTP decarboxylase (Ichiyama et al., 1968), so the former step is thought to be rate-limiting. For this reason, it seemed logical to investigate the activity of this enzyme to see how it affected levels of 5-HTP, the latter then serving as the substrate for 5-HTP decarboxylase. The synthetic pathway for 5-HT in terms of enzyme activity could then be assessed as an integated unit.

CHAPTER FIVE

The activity of TRY hydroxylase measured over 24 hours

5.1 Introduction

The first evidence of a specific TRY hydroxylase enzyme in normal mammalian tissue was reported by Grahame-Smith (1964). The enzyme was detected in homogenates of whole brain from dogs and rabbits but not in the high speed supernatant fraction from the centrifugate of the brain stem. Subsequent reports from several laboratories have also described the hydroxylation of TRY *in vitro* with extracts from various mammalian brain tissues (Gal, 1965; Gal *et al.*, 1966; Nakamura *et al.*, 1965; Lovenberg *et al.*, 1967; Jequier *et al.*, 1967; Grahame-Smith, 1967). This enzyme activity was also observed in intact cells of chromobacterium violaceum (Mitoma *et al.*, 1956), cell free extracts of carotinoid tumors (Lovenberg *et al.*, 1967) and mouse neoplastic mast cells (Levine *et al.*, 1964; Lovenberg, *et al.*, 1965).

The biosynthesis of the putative neurotransmitter, 5-HT involves two consecutive reactions, the first being the hydroxylation of TRY to form 5-HTP, which is catalysed by TRY hydroxylase. The 5-HTP is then decarboxylated to 5-HT (Udenfriend *et al.*, 1957). It is believed (though largely untested) that TRY hydroxylation may function as a rate-limiting step in 5-HT formation, because its maximum velocity is lower than that of the 5-HTP decarboxylase which controls the successive step (Ichiyama *et al.*, 1968). This is supported by the fact that endogenous levels of 5-HTP in the brain are very low (5-25 ng/g) making accurate estimations difficult (Linquist *et al.*, 1975).

5.1.1 Distribution

The anatomical distribution of TRY hydroxylase closely parallels the content of 5-HT in the rat brain (Saavedra, 1977). The enzyme is especially concentrated in the raphe nuclei, which contain large numbers of serotonergic cell bodies. The highest concentration among the raphe nuclei and also the highest for cells in the brain areas studied was found in the nucleus raphe dorsalis. There was a 43 fold difference between the concentration there and the lowest level measured which was in the cerebellar cortex. TRY hydroxylase is also concentrated in regions of the brain through which dense bundles of serotonergic axon terminals travel. Notable among these is the median forebrain bundle in the posterior hypothalamus. In the limbic system there are relatively high levels in specific areas such as the cingulate cortex, the nucleus tractus diagonalis, the nucleus septalis medialis and the nucleus amygdaloideus lateralis. In the mesencephalon, the nucleus interpeduscularis, the area tegmentalis ventralis, the colliculus superior and substantia grisea centralis are the areas with highest activities. Other brain areas with moderate enzyme activity are the locus coeruleus, the inferior olive, and the area postrema. In the pituitary gland, TRY hydroxylase activities are higher in the intermediate and posterior lobes.

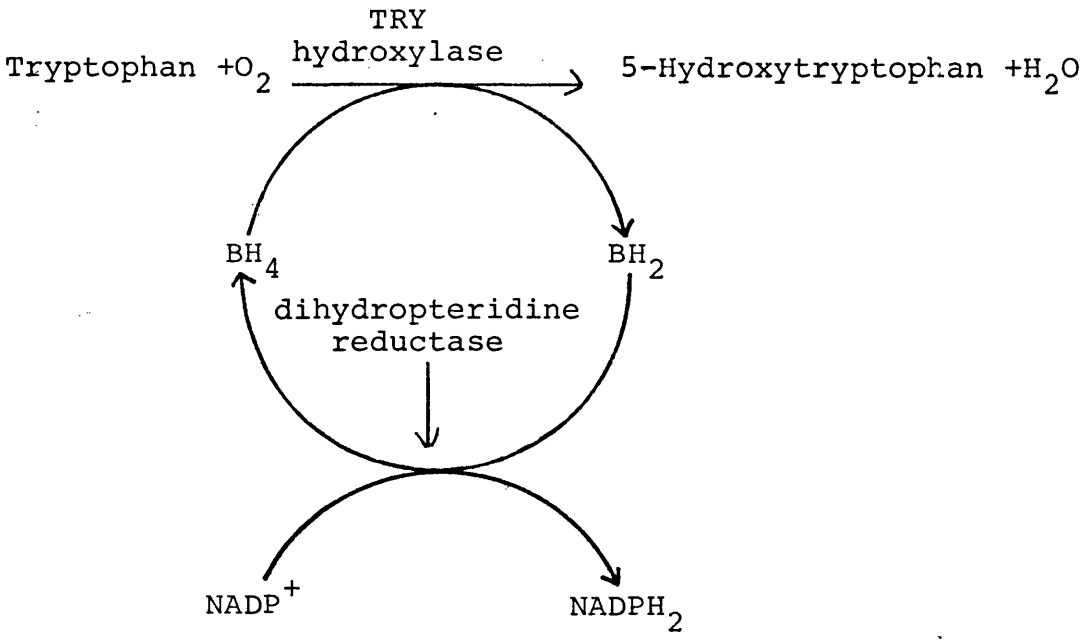
5.1.2 Co-factor requirements

Early work with TRY hydroxylase *in vitro* (Jequier *et al.*, 1967) showed that the Km for TRY using a synthetic cofactor 6, 7 dimethyl-tetrahydropterin ($DMPH_4$) was 300μM. This value is far above the plasma or brain concentration of TRY both of which are approximately 30μM for rats (Grahame-Smith, 1971; Fernstrom and Wurtman, 1971). This generated considerable speculative interest that the enzyme might not normally be saturated and that the overall rate of 5-HT synthesis might be partially dependent on the availability of TRY. Subsequent work however (Friedman *et al.*, 1972) showed that the Km for the enzyme varied significantly with the pterin cofactor used. The value in the presence of the naturally occurring cofactor tetrahydrobiopterin (BH_4) was 50μM which is more consistent with *in vivo* observations using TRY loading (Fernstrom and Wurtman, 1971) than the previously accepted value of 300μM. A dose of 50 mg/kg TRY intraperitoneally caused the brain TRY concentration to increase approximately three-fold (90μM) and the 5-HT concentration to double but further increases of brain TRY (up to 270μM) did not produce appreciable increases in the amount of 5-HT. Grahame-Smith (1971) noted a similar effect but found that the maximum rise in 5-HT occurred with a dose of 120 mg/kg TRY which produced a brain TRY concentration of approximately 350μM. Thus, 5-HT formation *in vivo* is maximal when the brain TRY concentration rises by 3-6 times (reaching 3-6 times the Km of TRY hydroxylase measured *in vitro* with BH_4 as the cofactor). A similar

conclusion was derived from estimates of the *in vivo* activity of TRY hydroxylase (Carlsson *et al.*, 1973b). This method involves the inhibition of 5-HTP decarboxylase with, for example, 3-hydroxybenzylhydrazine (NSD 1015). 5-HTP accumulates in the rat or mouse brain initially at a constant rate. Efflux is slow so accumulation of this intermediate essentially reflects its rate of formation and should serve as an indicator of the *in vivo* activity of the hydroxylase. It was found that TRY hydroxylase was slightly less than half-saturated with its amino acid substrate. The TRY content of the brain had to be increased by several times its normal level before the maximum rate of hydroxylation was observed. The Km calculated was 60μM. Inhibition by high concentrations of TRY was not observed. Thus, it appears that TRY hydroxylase is not saturated *in vivo* and that by changing the brain TRY concentrations it is possible to control the rates of 5-HT synthesis and accumulation.

5.1.3 *Stoichiometry of the reaction*

Friedman *et al.*, (1972) showed that with tetrahydropterin (BH_4) 1 mole of 5-HTP was formed for each mole of BH_4 oxidised. This result, taken together with their demonstration that hydroxylation is stimulated by dihydropteridine reductase led to the reaction being formulated as in the diagram below:-



They also showed that the pterin functioned as a cofactor i.e. it functioned catalytically during hydroxylation. The pterin cofactor can only function in the reduced form and dihydropteridine reductase, which has been isolated and purified from liver homogenate (Craine et al., 1972) can maintain the cofactor in this state. The enzyme is also present in high concentrations in liver, kidney, brain and adrenal medulla. This reductase might be regulatory for the hydroxylation of TRY (Musacchio et al., 1971) but such a possibility is not substantiated by the available data (Craine et al., 1972). It would seem that the amount of reduced BH_4 rather than the reductase could perform a regulatory function. The concentration of BH_4 in the brain is estimated to be 0.75 mg/g (3μM if evenly distributed: Guroff et al., 1967), but inferences as to the regulatory potential for the biosynthesis of 5-HT in brain are premature until more is known about the regional and cellular distribution of BH_4.

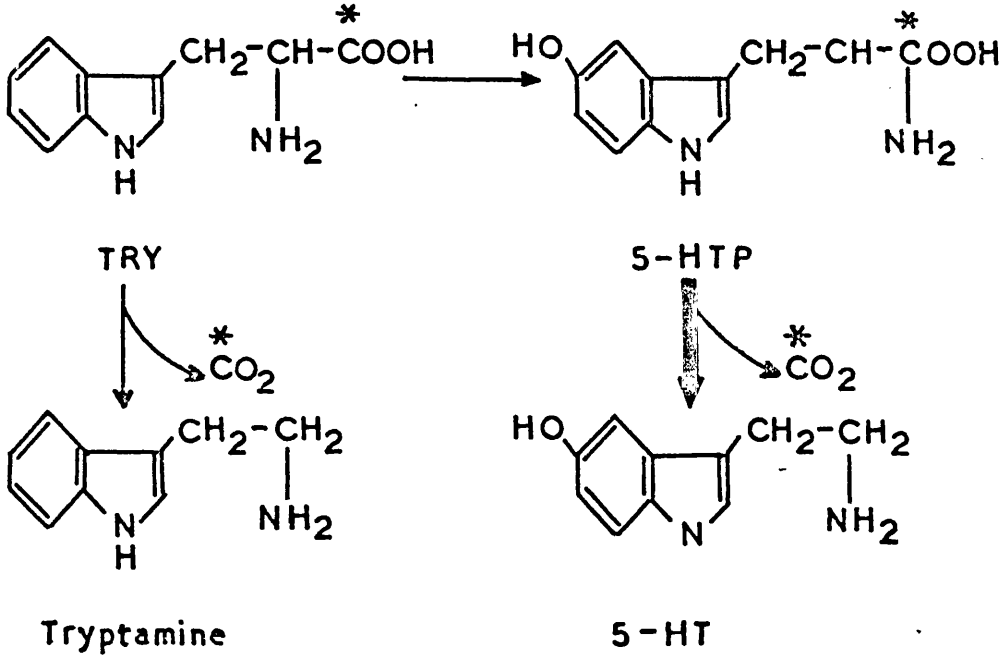
Cofactor requirements vary according to whether a synaptosomal or soluble enzyme preparation is used. With a synaptosomal preparation BH_4, $DMPH_4$, $NADH_2$, ascorbic acid, Fe^{2+} ions or sulphydryl compounds e.g. β-mercaptoethanol or dithiothreitol did not stimulate the hydroxylase activity significantly (Ichiyama et al., 1970; Friedman et al., 1972). Presumably the reductant in this system is generated in situ by some endogenous substance which is unknown at present. The enzyme activity in the supernatant fraction showed an absolute requirement for BH_4 and was stimulated by reduced pyridine nucleotide, dihydropteridine reductase, a high concentration of β-mercaptoethanol or dithiothreitol and catalase. NADPH could substitute at least in part for the sulphydryl reagent but not the reduced pteridine. Fe^{2+} ions also stimulated the reaction but only in the absence of catalase; this finding suggests that catalase or Fe^{2+} protects a sensitive component of the TRY hydroxylase system from inactivation of hydrogen peroxide (Friedman et al., 1972). Ichiyama et al., (1970) measured the Km for the different preparations and found the Km for the soluble TRY hydroxylase to be approximately 100μM while that for the synaptosomal enzyme was approximately 20μM. This led to the suggestion that two different TRY hydroxylases, characterised by different Km values for TRY may be present in the brain. These two values are close to those observed by Friedman et al., (1972) for TRY in the presence of $DMPH_4$ and BH_4 respectively.

In the first experiment the soluble enzyme was assayed in the presence of $DMPH_4$ and the synaptosomal preparation in the presence of endogenous cofactor. Since the brain pterin cofactor is probably identical with, or closely related to BH_4 perhaps the differences in Km values are not characteristic of a soluble and particulate enzyme but are characteristic of the pterin cofactor by which the hydroxylase is functioning (Friedman et al., 1972).

The Km for oxygen is also strongly influenced by the pterin cofactor used. In the presence of BH_4 the Km was found to be 2.5% which is less than the oxygen concentration of 5% in the brain of animals breathing air (Jamieson and Van den Brenk, 1965). It seems likely therefore that the enzyme is not normally limited by oxygen supply. This conclusion contrasts greatly with the one that would have been reached if the Km for oxygen of 20%, determined in the presence of the artificial cofactor $DMPH_4$, was relied on (Friedman et al., 1972). The sensitivity to inhibition by high concentrations of TRY is apparent with BH_4 as cofactor but not with $DMPH_4$ (up to 2 mM TRY).

5.2 Method

The method is an adaptation of that described by Ichiyama et al., (1970). It involves no separations and utilizes the differing affinity of 5-HTP decarboxylase for two amino acids, 5-HTP and TRY. The Km of 5-HTP (approximately



3 to 14 mM), so that in a mixture of TRY and 5-HTP at low concentrations, the latter compound will be decarboxylated preferentially.

5.2.1 Preparation of the synaptosomal sample

Male CFY rats (180-220g) were stunned and decapitated. The brains were rapidly removed and the pineal gland discarded. Following weighing, the brains were homogenised in ice-cold 0.32M sucrose using a teflon pestle and glass tube homogeniser with 0.01" radial clearance to form a 10% homogenate. The latter was centrifuged at 1,000 x g for 10 mins at 4°C. The resulting supernatant was decanted off and centrifuged at 12,000 x g for 20 mins.

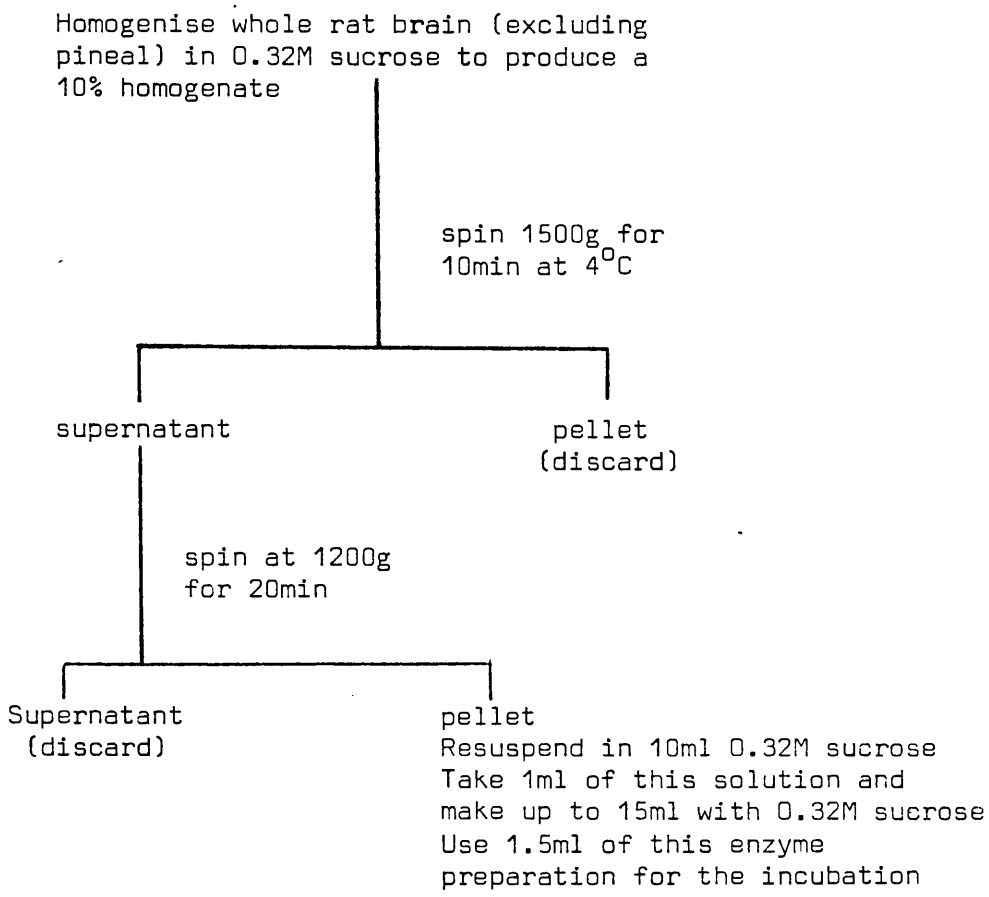
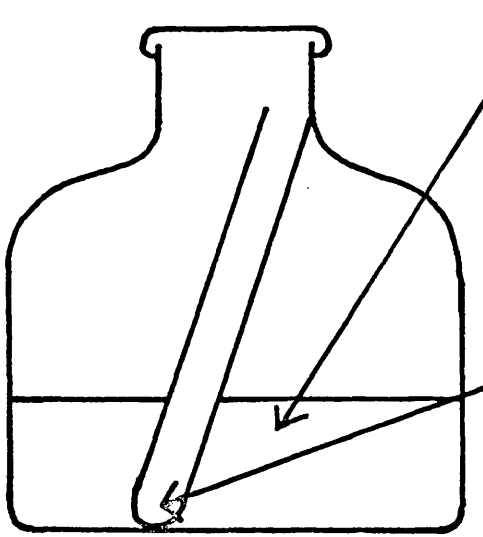


Fig.31 Method for the measurement of tryptophan hydroxylase activity

1 Pre-incubate the following for 10min at 37°C
500umol Tris acetate buffer (pH 8·1)
1·25mmol sucrose
1·5ml enzyme preparation
(Final concentrations)

2 Add 25nmol L-TRY + 6·7 nmol C^{14} DL-TRY
Seal the bottle



3 1ml 4N $HClO_4$

4 Inject 0·5ml 0·1 hyamine into the micro test-tube. Leave for a further 3hr at 37°C

5 Transfer an aliquot of hyamine for scintillation counting

Fig. 32. Incubation Procedure

The pellet formed was resuspended in 10 ml 0.32 M sucrose; 1ml of which was made up to 15 ml with 0.32M sucrose (i.e. 150 dilution of the original pellet). This synaptosomal preparation was used for the enzyme assay. A summary of this method is shown in Fig. 31.

5.2.2 Incubation

Tris acetate buffer pH8.1 (500μmol) and 1.25 mmol sucrose were pre-incubated with 1.5 ml of the enzyme preparation in a 15 ml narrow neck bottle (Fig. 32) for 10 mins at 37°C. 25 nmol L-TRY + 6.7 nmol DL-{carboxyl -^{14}C} TRY (444,000dpm) was injected into the reaction mixture whose final volume was 5 mls. The final concentration of L-TRY was 5.67 x 10^{-6}M. A micro test-tube was placed inside the bottle and the latter was clamp sealed. The reaction proceeded for 30 mins and was terminated by the injection of 1 ml 4N $HClO_4$ into the bottle. The acid also serves to release the $^{14}CO_2$ produced from solution. 0.5 ml of 0.1M hyamine hydroxide (diluted with n-butanol) was injected into the micro test-tube and the incubation was continued for a further three hours to allow complete absorption of the released $^{14}CO_2$ by the hyamine. Hyamine is a high-molecular weight quaternary amine. Passman et al., (1956) were the first to use this substance for collecting $^{14}CO_2$. It is toxic towards most biological systems which is why it is necessary to add the organic base at the end of the incubation period.

Fig. 33 The effect of varying the enzyme concentration on hydroxylase activity at a final L-TRY concentration of 1.067×10^{-5}M.
37.5ml, 75ml, 150ml dilution of P_2 pellet with 0.32M sucrose.
Conditions of incubation - pH8, 37°C, 0-60 min; 500µmol Tris acetate buffer, 1.25 mmol sucrose, 1.5ml enzyme prep^{n} 50nmol L-TRY + 6.7nmol DL^{14}C-TRY (final conc^{n}s) in total vol. of 5ml.
Velocity expressed as nmols 5-HT produced/g wet brain weight/hour.

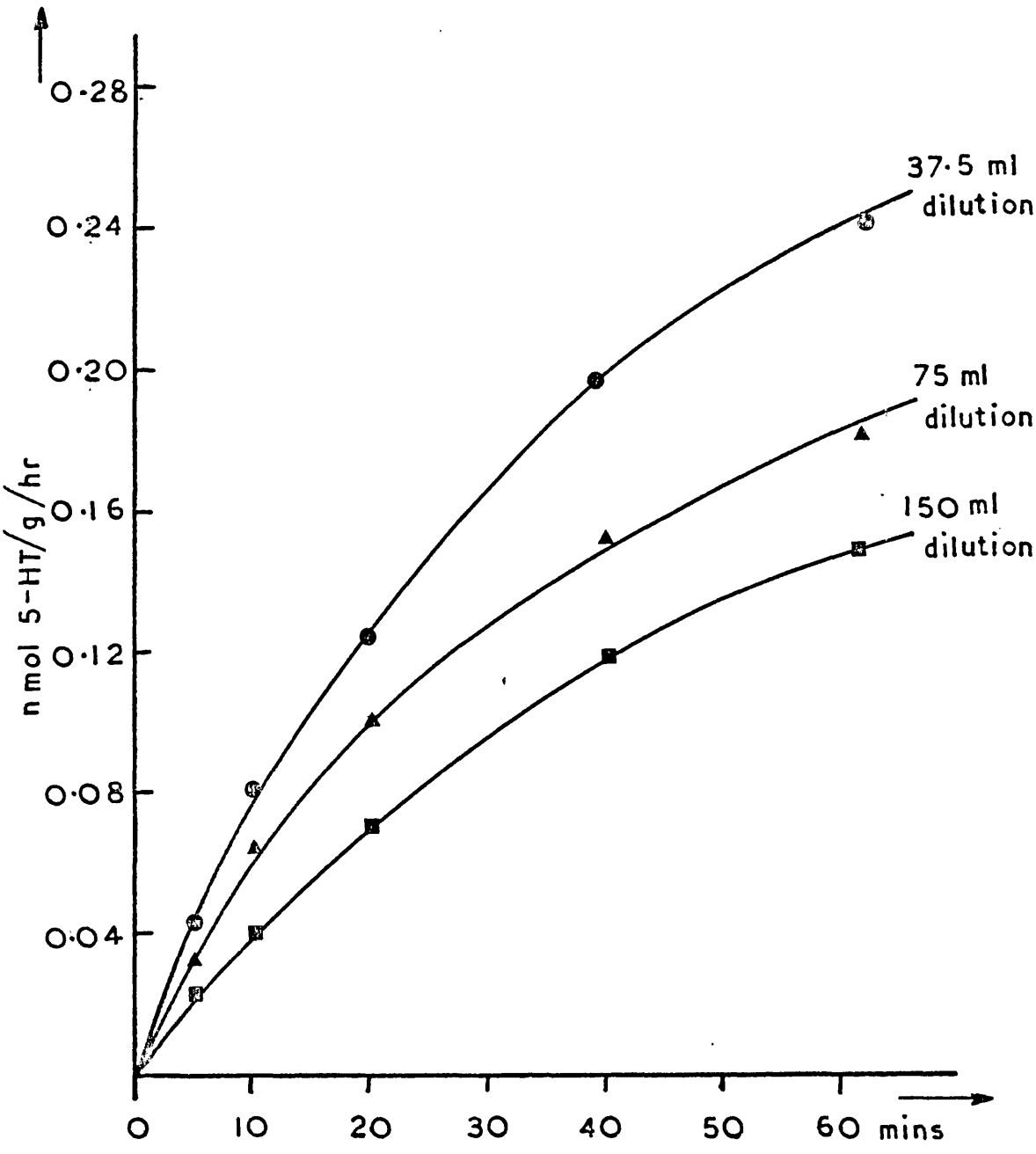

0·28
0·24
0·20
0·16
0·12
0·08
0·04
n mol 5-HT/g/hr
37·5 ml dilution
75 ml dilution
150 ml dilution
0
10
20
30
40
50
60 mins

Fig. 33

Fig. 34 The effect of varying the enzyme concentration on hydroxylase activity at a final L-TRY concentration of $1.007\ 10^{-4}$M. 37.5ml, 75ml, 150ml dilution of P_2 pellet with 0.32M sucrose. Conditions of incubation - pH8, 37°C, 0-60 min; 500umol Tris acetate buffer, 1.25mmol sucrose, 1.5ml enzyme prep^n, 50nmol L-TRY + 6.7nmol DL^{14}C-TRY (final conc^{n}s) in total vol. 5ml. Velocity expressed as nmols 5-HT produced/g wet brain weight/ hour.

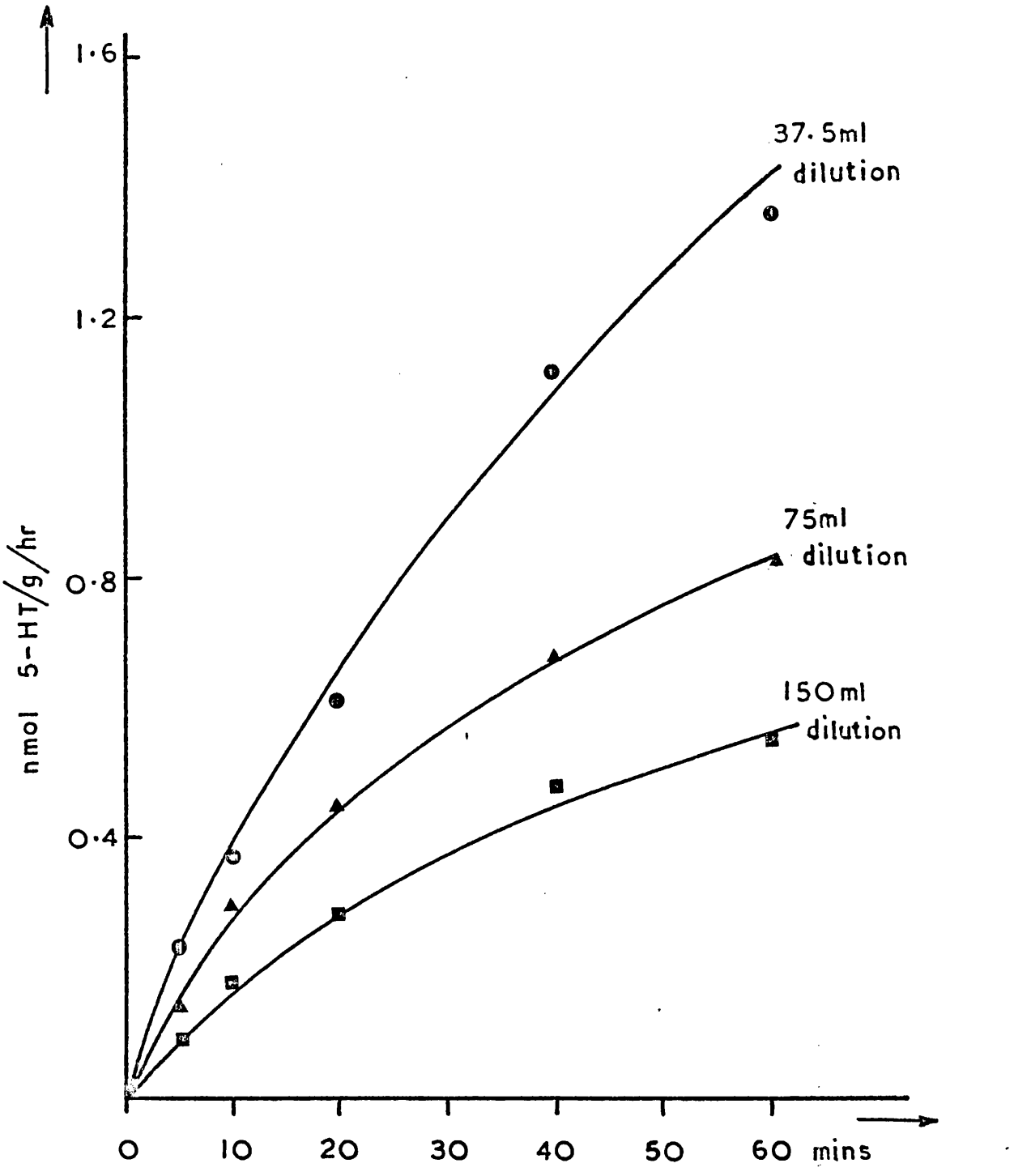

1·6
1·2
0·8
0·4
nmol 5-HT/g/hr
37·5ml dilution
75ml dilution
150ml dilution
0
10
20
30
40
50
60 mins

Fig. 34

Fig. 35 The effect of varying the substrate concentration on TRY hydroxylase activity.
Conditions of incubation - pH8, 37°C, 0-60 min; 500µmol Tris acetate buffer, 1.25 mmol sucrose, 1.5 ml of x150 dilution of enzyme prep^{n}, L-TRY 5.067×10^{-5}M, 1.067×10^{-5}M, 5.67×10^{-6}M (final conc^{n}s) in total vol. of 5ml.
Velocity expressed as nmoles 5-HT produced/g wet brain weight/hour.

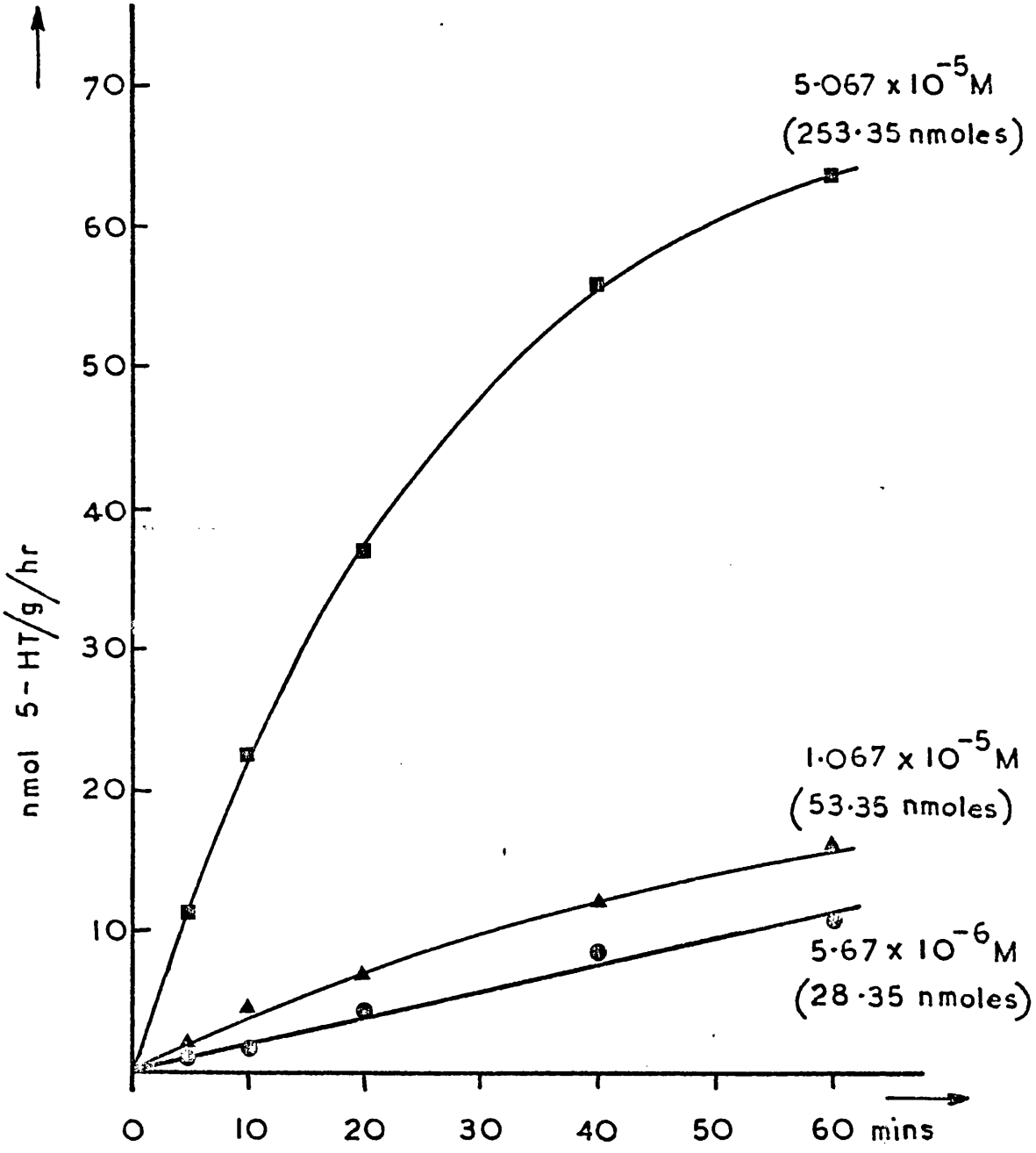

nmol 5-HT/g/hr
70
60
50
40
30
20
10
0
10
20
30
40
50
60 mins
5·067 x 10⁻⁵M
(253·35 nmoles)
1·067 x 10⁻⁵M
(53·35 nmoles)
5·67 x 10⁻⁶M
(28·35 nmoles)

Fig. 35

Fig. 36 The effect of altering the substrate concentration (1.067×10^{-5}M, 5.67×10^{-6}M L-TRY) on TRY hydroxylase activity.
Conditions of incubation - refer to Fig. 35.

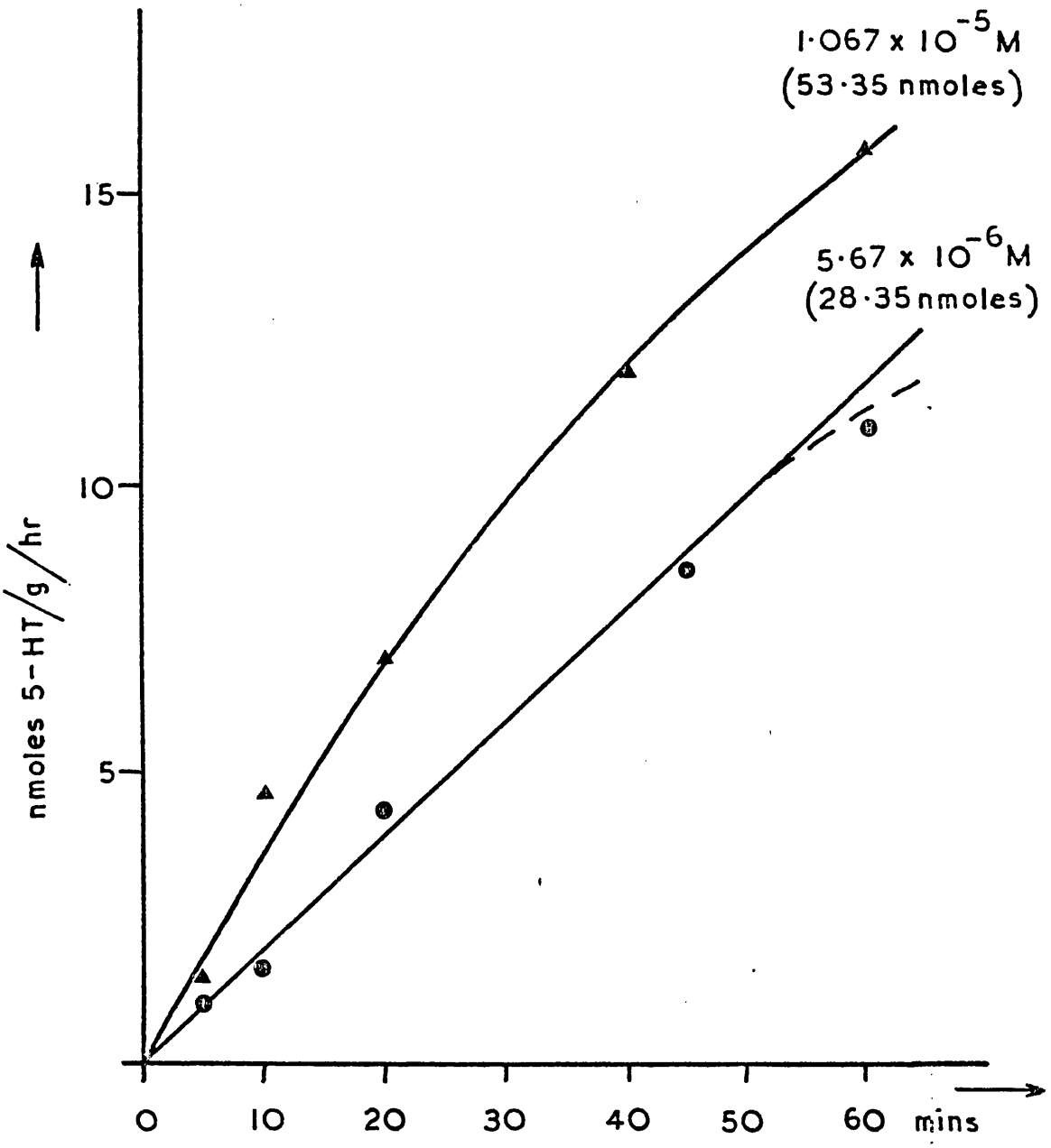

1·067 x 10⁻⁵M
(53·35 nmoles)
5·67 x 10⁻⁶M
(28·35 nmoles)
15
10
5
nmoles 5-HT/g/hr
0
10
20
30
40
50
60 mins

Fig. 36

The seal was removed from the bottle and an aliquct of hyamine transferred to a vial containing 10 ml of Triton-xylene scintillant for counting. Blanks were prepared by injection of the acid into the bottle before addition of the substrate.

This assay procedure was used to determine the activity of TRY hydroxylase over 24 hours. In the results section the way in which variables within the method were altered to achieve these optimal conditions is described and experiments were also carried out to assess the sensitivity of the method.

5.3 Results

Expt. 1 The effect of varying the enzyme concentration. Three dilutions of the original pellet were used (37.5 ml, 75 ml, 150 ml) and two final substrate concentrations (1.067×10^{-5}M and 1.007×10^{-4}M L-TRY). The incubation was carried out for 5, 10, 20, 40 and 60 min. The results (Figs. 33 and 34) show that at the dilutions and concentrations used the formation of 5-HT did not proceed at a constant rate nor did it double as the pellet dilution halved.

Expt. 2 The effect of varying the substrate concentration. Using the 150 ml pellet dilution and the following final concentrations of L-TRY:- 5.67×10^{-6}M, 1.067×10^{-5}M and 5.67×10^{-5}M, the enzyme activity was measured over 60 mins. The results (Figs. 35 and 36) show that only

Table 3

Inhibition of tryptophan hydroxylase by p-chlorophenylalanine.

4 exp. rats - 400 mg/kg PCPA 24 hours prior to sacrifice

4 control rats - saline

X 150 pellet dilution 5.67 x 10^{-6}M L-TRY

Incubated 37°C for 30 mins at pH 8.1

	nmol $^{14}CO_2$ produced/g/hr
	8.86
	9.77
	8.88
	8.73
Mean	9.06
Experimental	5.01
	6.06
	5.89
	4.83
Mean	5.45

The experimental animals showed a 39.74% reduction in tryptophan hydroxylase activity.

at 5.67×10^{-6}M did 5-HT formation achieve a constant rate and be half that obtained at x2 the substrate concentration. This pellet dilution and substrate concentration were chosen for the final experiment.

Expt. 3 Estimation of the Km and Vmax for TRY hydroxylase.
A rough estimate of the Km and Vmax values of the enzyme were calculated by using substrate and velocity values obtained from experiments 1 and 2 and constructing a Lineweaver-Burk plot. The Km was calculated as 61.5μM and Vmax 125 nmol/g/hr.

Expt. 4 Inhibition of TRY hydroxylase by p-chlorophenylalanine (PCPA).
This experiment was carried out to verify the fact that the method was measuring the activity of TRY hydroxylase and not an artefact. Experimental rats were injected intraperitoneally with 400 mg/kg PCPA (100 mg/ml PCPA hydrochloride buffered to pH5.5 with 0.5N Na OH) 24 hours prior to sacrifice. Control rats were injected with 0.9% saline. As the results show (Table 3), there was an approximately 40% reduction in TRY hydroxylase activity in the experimental rats.

Expt. 5 The effect of varying the specific activity of the substrate.
A 150 ml pellet dilution was used and the final substrate concentration (L-TRY) was 5.34×10^{-6}M. The following

Fig. 37 The effect of altering the specific activity of the substrate on TRY hydroxylase activity.
Conditions of incubation - pH8, 37°C, 30 min; 500µmol Tris acetate buffer 1.25 mmol sucrose, 1.5ml of x 150 dilution of enzyme prep^{n} altered ^{14}C DL-TRY conc^{n} (0.025 µci⟶0.4 µci) within total L-TRY final conc^{n} of 26.7 nmols.

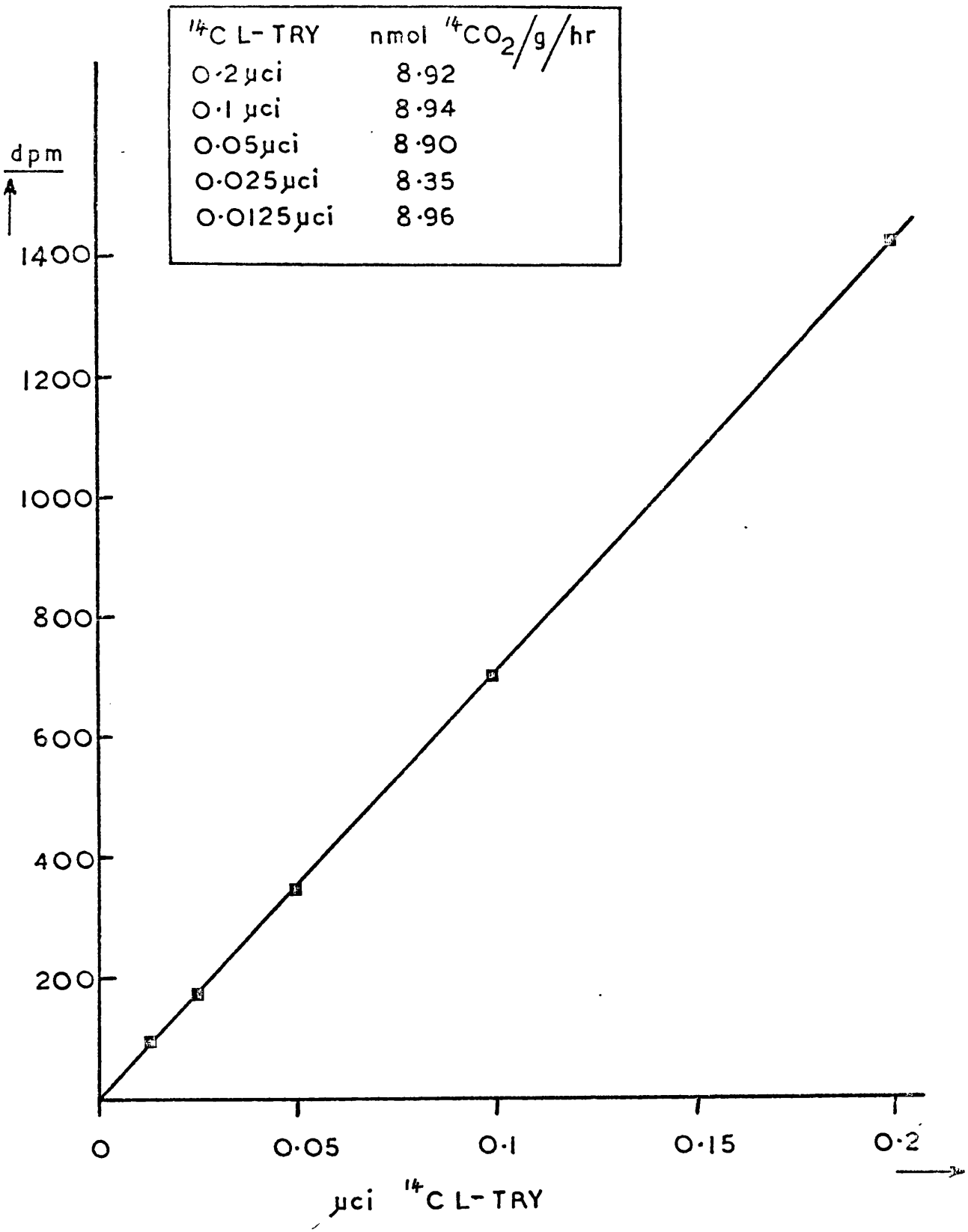

^{14}C L-TRY nmol $^{14}CO_2$/g/hr
0·2 µci 8·92
0·1 µci 8·94
0·05µci 8·90
0·025µci 8·35
0·0125µci 8·96
dpm
1400
1200
1000
800
600
400
200
0
0·05
0·1
0·15
0·2
µci ^{14}C L-TRY

Fig. 37

Table 4

Comparison of acid to heat inactivated blank values

1 Animal : x 150 pellet dilution:

L-TRY 5.67 x 10^{-6}M. Incubate at 37°C for 30 mins at pH 8.1.

Dpm (acid blank)	Dpm (heat inactivated blank)
895	261
893	286
890	305
879	273
889	281

Value of the heat-inactivated blank is 31.6% that of the acid blank.

Table 5

Method variation

8 animals: X 150 pellet dilution: L-TRY concentration

5.67×10^{-6}M (222,000dpm). Incubate at 37°C for 30 mins at pH 8.1.

	nmol $^{14}CO_2$ produced/g/hr
	6.86
	7.24
	6.96
	7.20
	7.22
	6.92
	6.40
	6.90
Mean	6.96
Variance	0.0665
S.D	0.258
Coeff. of variance	0.955%

Table 6

Animal variation

8 animals : x 150 pellet dilution: L-TRY

5.67×10^{-6}M (222,000 dpm). Incubate 37°C for 30 mins pH 8.1

	nmols $^{14}CO_2$ produced/g/hr
	7.00
	7.90
	6.44
	7.32
	6.60
	6.90
	7.42
	6.84
Mean	7.05
Variance	0.197
S.D	0.444

Coeff. of variance $\left(\frac{\text{S.D}}{\text{Mean}} \times 100\right) = 2.79\%$

amounts of DL-^{14}C TRY were used 0.025, 0.05, 0.1, 0.2 and 0.4 μci (i.e. the effective L-TRY amounts are half these values). As seen from Fig. 37 an increase in specific activity produced a proportional increase in d.p.m., which was linear over the range investigated. This ensured that there was no deleterious effect in altering the amount of radioactive substrate used in these experiments.

Expt. 6 A comparison of acid-inactivated and heat-inactivated blanks.

Table 4 shows the blank values were reduced by 32% when the enzyme preparation was destroyed by boiling rather than acid.

Expt. 7 Method and animal variation.

Tables 5 and 6 show the coefficient of variance for the method and animal variation to be respectively 0.955% and 2.79%. These figures were used to calculate the smallest variation (0.55 nmol/g/hr) in enzyme activity detectable with the method used.

Having established the appropriate conditions for estimating the activity of TRY hydroxylase, the enzyme's activity was measured at 6 clock hours. Groups of 8 rats housed for two weeks under the conditions described in Chapter 2 were sacrificed at 4 hourly intervals. The brains were rapidly removed and assayed as described in Section 5.2.

Fig. 38 The 24-hour variation in TRY hydroxylase activity. Conditions of incubation - pH8, 37°C, 30 min; 500 µmol Tris acetate buffer, 1.25 mmol sucrose, 1.5 ml of x 150 dilution of enzyme prep^{n}, 5.67 x 10^{-6}M L-TRY (25 nmol L-TRY + 6.7 nmol ^{14}C DL-TRY) final conc^{n}s. n = 8 at each clock hour. Velocity expressed as nmoles 5-HT produced/g wet weight/hour.

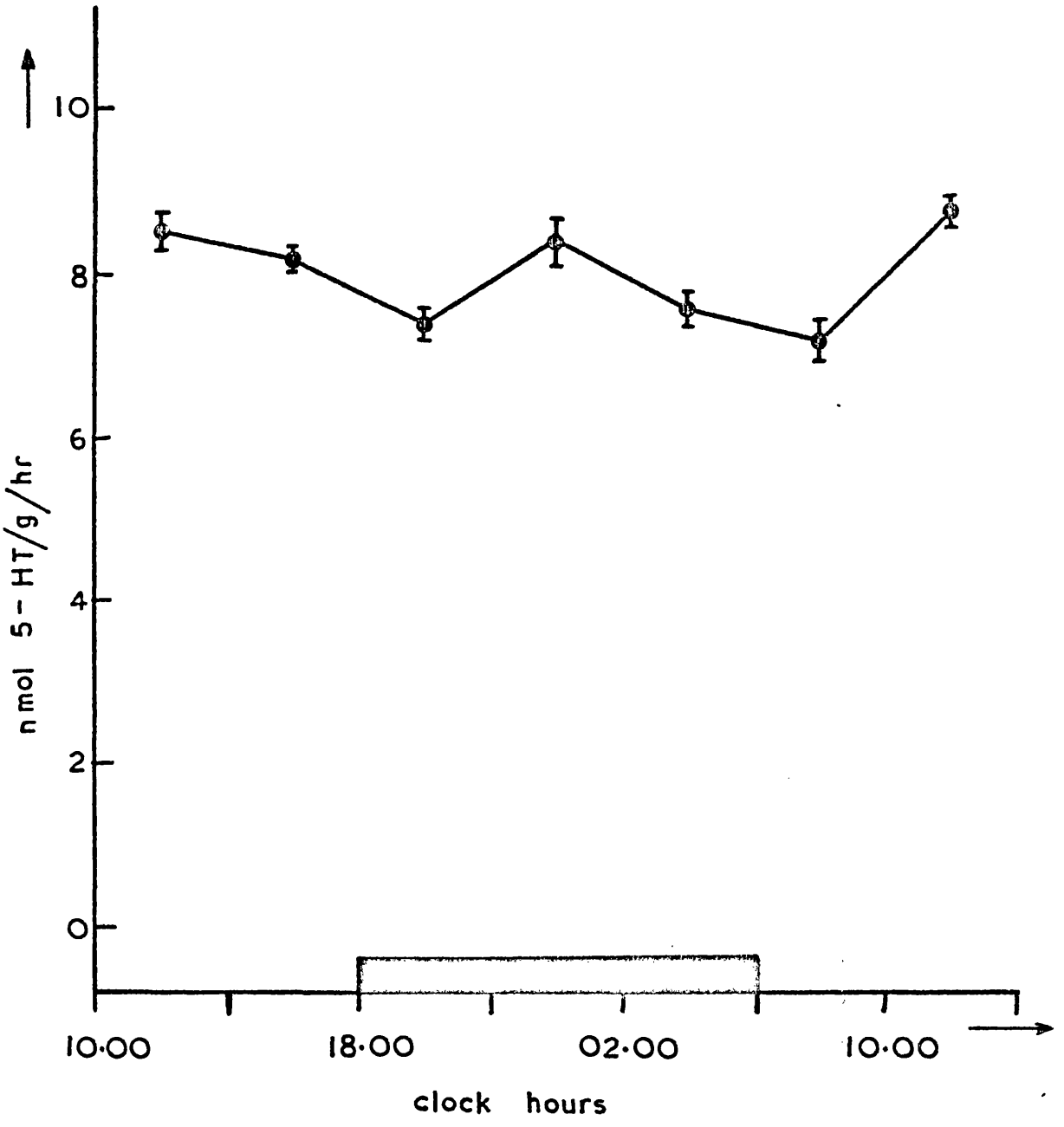


Fig. 38

The 24-hour variation of TRY hydroxylase activity

As seen from Fig. 38 no circadian rhythm in TRY hydroxylase activity was found to exist in the rat brain.

5.4 Discussion

Early methods used to determine the *in vitro* activity of TRY hydroxylase were based on the determination of radioactive 5-HT, which was separated from a large excess of unreacted TRY by paper chromatography, paper electrophoresis or ion-exchange resins. Various methods have been developed in an attempt to overcome these lengthy separations such as direct determination of 5-HTP (Friedman *et al.*, 1972), a tritium release assay (Lovenberg *et al.*, 1971), a carbon dioxide trapping method (Ichiyama *et al.*, 1970) and the formation of melatonin (Saavedra *et al.*, 1973).

The method involving the direct determination of 5-HTP measured the enzyme activity in the synaptosomal fraction and also the soluble fraction after a 10-fold purification of the enzyme from rabbit hindbrain. The purification involved on extraction followed by adsorption and elution from calcium phosphate, then ammonium sulphate fractionation. The enzyme samples could be stored under nitrogen at -80° in small aliquots after being quick dried. Incubation was carried out at 25 mins at $37^{\circ}C$ and the reaction stopped with 70% $HClO_4$. After centrifugation, the 5-HTP was determined in the supernatant fluorimetrically (in concentrated HCl emission 538 nm excitation 295nm).

Table 7

24 hour variation in tryptophan hydroxylase activity

Clock Hour	nmol $^{14}CO_2$ produced/g/hr
12.00	8.58 ± 0.24
16.00	8.21 ± 0.16
20.00	7.42 ± 0.28
24.00	8.45 ± 0.31
04.00	7.64 ± 0.22
08.00	7.17 ± 0.31
12.00	8.84 ± 0.18

X 150 pellet dilution:

5.67×10^{-6} M L-TRY. Incubated at 37°C for 30 mins at pH 8.1. Each result is mean of 8 animals.

This method is based on the fact that, under defined conditions 5-HTP fluoresces whereas TRY does not (Udenfriend, 1962). This method was not found to be particularly sensitive when tested in our laboratories.

The method of Lovenberg et al., (1971) measures the activity of TRY hydroxylase using {5-^{3}H} TRY as substrate. When hydroxylation occurs at the 5 position on the indole ring, the tritium shifts to the 4 position. Subsequent acidification of the reaction mixture causes the release of tritiated water. This assay requires the chemical synthesis and purification of {5-^{3}H} TRY, which is time consuming (the purification involves two chromatographic separations on an amino acid analyser), and the resulting product is stable for only 3 to 4 weeks.

The method of Saavedra et al., (1973) is based on the conversion of TRY to 5-HT and the conversion of the latter to {^{3}H} melatonin by a two step reaction involving the N-acetylation of 5-HT to form N-acetyl 5-HT, followed by the O-methylation of N-acetyl 5-HT to form melatonin, utilizing {^{3}H} methyl-S-adenosyl-L-methionine ({^{3}H} -SAM) as the methyl donor. {^{3}H} melatonin is isolated from the {^{3}H} SAM by a simple organic extraction. Under the conditions of the assay {^{3}H} melatonin is the only radioactive product isolated in detectable amounts. The assay requires the preparation of partially purified N-acetyltransferase, 5-HTP decarboxylase and hydroxyindole-O-methyltransferase. Although the method is a sensitive

radio-enzymatic microtechnique for measuring 5-HT and its synthesising enzymes in discrete brain regions, it involves intricate multi-step reactions and is not a simple assay to establish.

The advantages of the carbon dioxide trapping method are that no separation is required so a simple and rapid method is available. It is also reliable as the sensitivity is relatively high and there is no possibility of contaminating $^{14}CO_2$ with other radioactive materials. The disadvantages are that it cannot be applied to crude fractions from tissues such as liver where alternative pathways are known to exist for the catabolism of TRY. Also, it is apparent that in a reaction mixture containing relatively high concentrations of TRY an appreciable amount of CO_2 evolved will be due to tryptamine formation instead of 5-HT formation. Therefore substrate concentrations above the K_m should not be used for the assay. For these reasons the last method described was chosen for this work. As the assay was performed on rat brain and low substrate concentrations were used (the concentration in the standard assay was 5.67×10^{-6}M), the main disadvantages of the method did not apply. Further a synaptosomal preparation was used so the addition of cofactor, partially purified 5-HTP decarboxylase, and reagents necessary to assay the soluble fraction as described previously were not required.

Certain unexpected problems were encountered, these being the relatively high blank values and the loss of linearity

in 5-HT production with time except at the dilution and substrate concentration used in the standard method. The high blank values varied only when the specific activity of the substrate varied. There was a 32% reduction in the blank value if inactivation of the enzyme was achieved by boiling rather than with acid. An explanation is that the acid itself is cleaving $^{14}CO_2$ from the substrate causing a non-enzymatic production. Under normal conditions an incubation cannot be terminated by placing the sealed bottle in boiling water, thus acid must be used.

The loss of linearity of 5-HT production with time could be explained by end-product inhibition, a lack of oxygen, deterioration of the enzyme preparation with time or cofactor availability. In vitro, end-product inhibition appears not to be a significant regulatory mechanism in the serotonergic system. It has been shown that 5-HT inhibits the enzyme only in high concentrations (greater than 10^{-3}M) and then in a non-competitive way (Jequier et al., 1969; Knapp and Mandell, 1973) even with the more effective cofactors. In vivo, however, Hamon et al.,(1973) have demonstrated that manoeuvres intended to increase (e.g. MAO inhibition) or decrease (e.g. reserpine induced depletion) intraneuronal 5-HT led to a decrease and increase in total 5-HT, respectively. 5-HT synthesis was reduced when intraneuronal levels of the amine reached 2.5 times normal levels following MAO inhibition (Macon et al., 1971) Carlsson et al., (1973b) observed a decrease in the rate of

5-HTP accumulation induced by a decarboxylase inhibitor in mice injected with a MAO inhibitor. The decrease was not observed when inhibitors of the two enzymes were given simultaneously but only when the MAO inhibitor was given beforehand, leading to an increased 5-HT level. Also the two hallucinogenic agents LSD-25 and dimethyltryptamine caused a reduction of the 5-HTP accumulation. These agents are believed to activate postsynaptic receptors. Thus conflicting results regarding the role of end-product feedback inhibition exist. In vitro experiments suggest that 5-HT is not a probable regulatory molecule for modulating TRY hydroxylase activity because too great a concentration of 5-HT is required to do so, and the inhibition is non-competitive. The experiments in vivo however, suggest that treatments that increase extravesicular, intraneuronal 5-HT decrease 5-HT biosynthesis and that treatments that decrease the levels have an opposite effect.

As the circadian rhythm of brain TRY is out of phase with that for 5-HT and as evidence suggests TRY hydroxylase is the rate-limiting step in the formation of 5-HT, it seemed a logical step to assay the activity of the enzyme over 24 hours. If evidence of variation in the enzyme activity was apparent, it might indicate an important regulatory step in controlling 5-HT levels. As seen from the results, however, no variation in activity was observed. These results differ from those of Shibuya et al., (1978) who found a clear 24-hour rhythm in TRY hydroxylase

activity of the pineal body. In the latter, 5-HT shows a rhythm (Quay, 1963) with the opposite phase of the marked circadian rhythms of N-acetyltransferase (Klein et al., 1970), N-acetylserotonin (Klein et al., 1970) and melatonin (Ralph et al., 1971). A suggestion was made that pineal TRY hydroxylase in the rat may have a different value from that in the brain (Deguchi and Barchas, 1973) and these results would tend to substantiate these findings.

Having been unable to find any variation in enzyme activity connected with clock hour using an in vitro method, it was decided to continue investigation of factors controlling the 24-hour variation of 5-HT by using a behavioural model. By using in vitro methods the possibility of investigating short-term regulation via nerve stimulation (e.g. feedback control of synthesis via a neuronal loop as opposed to local end-production inhibition) is not feasible. It was thought that by using a behavioural model, which represented activation of central serotonergic receptors by enhancing the 'availability' of 5-HT at post-synaptic receptor sites, and to study this over 24 hours might provide more evidence as to how the activity of serotonergic neurons are regulated.

CHAPTER SIX

The 24-hour variation in TRY-induced hyperactivity in rats

6.1 Introduction

When rats are treated with a combination of monoamine oxidase inhibitor (MAOI) and L-TRY, they develop a syndrome of hyperactivity, hyperpyrexia, reciprocal forepaw treading, head weaving and hind-limb abduction (Hess and Doepnfer, 1961; Grahame-Smith, 1971a). Grahame-Smith has elucidated the neurochemical mechanisms underlying this syndrome and used it to investigate agents of potential clinical value in the treatment of neurological and psychiatric disorders, in which disturbances of endogenous central 5-HT levels have been implicated.

Biochemical analysis showed an increasing accumulation of brain 5-HT with increasing L-TRY doses up to 120mg/kg L-TRY and a brain TRY concentration of approximately 70µg/g brain weight. Above this dose and concentration no further increase in brain 5-HT accumulation occurred (Grahame-Smith, 1971a). Monoamine inhibition, L-TRY administration and intact TRY hydroxylase and 5-HTP decarboxylase activity were all shown to be essential prerequisites for the production of the behavioural syndrome (Grahame-Smith, 1971a). Indices of hyperactivity correlated with the amount of brain 5-HT accumulating in 1 hour after L-TRY loading but not with the absolute concentration of brain 5-HT, suggesting hyperactivity was dependant upon the rate of 5-HT synthesis. Reserpine and tetrabenezine, which both inhibit the uptake of and storage of 5-HT by intraneuronal granules (Pletscher et al., 1968; Carlsson, 1966), speeded the onset and rate of development of the hyperactive state without altering the

the synthesis of brain 5-HT. These results led Grahame-Smith (1971a) to suggest that when MAO is inhibited and the rate of 5-HT synthesis is increased, granular storage of 5-HT and other rate-limiting mechanisms for 5-HT, inactivation are unable to prevent 5-HT 'spilling over' to produce hyperactivity. Evidence for this syndrome being mediated by serotonergic neurons was further substantiated by the use of the hallucinogenic drug, 5-Methoxy-N,N-dimethyltryptamine (5 MeODMT), which is a 5-HT post-synaptic receptor agonist (Ahlborg et al., 1968). This drug produced a syndrome of hyperactivity, which was qualitatively identical to that produced by an MAOI and L-TRY but had a more rapid onset and was of shorter duration (Grahame-Smith, 1971b). The similarity of the syndromes suggested that 5-HT and 5 MeODMT were acting at the same sites. PCPA did not inhibit the behavioural effects of 5 MeODMT unlike those produced by an MAOI and L-TRY and this was indirect (but not absolute) evidence against 5 MeODMT acting through the release of endogenous brain 5-HT.

This work led to the proposal that the syndrome represented a model for 5-HT receptor activity. However, this conclusion was questioned when certain observations were reported to implicate the possible involvement of DA in the expression of the syndrome.

It was found that an MAOI plus L-DOPA produced a similar though not identical excitatory behaviour (Jacobs, 1974; Green and Grahame-Smith, 1974). Because of the overt similarity in these 2 syndromes, Jacobs (1974) proposed

that they might be dependant upon a common neurotransmitter. He tested this theory by observing whether blockade of either 5-HT or DA biosynthesis or their respective receptors was capable of blocking the production of the syndrome produced by precursors of the other transmitter. He found that pretreatment with α-methyl-ρ-tyrosine (α-MPT), a tyrosine hydroxylase inhibitor, failed to affect either the syndrome produced by an MAOI and L-TRY or that produced by an MAOI and L-DOPA, whereas PCPA blocked both. Similarly pimozide, a DA receptor antagonist had no effect on either syndrome while cinanserin and methysergide, 5-HT receptor antagonists abolished or markedly diminished both syndromes equally. Jacobs (1974) concluded that the effect of L-DOPA was mediated by 5-HT rather than DA and suggested that as L-DOPA increased the efflux of 5-HT from brain slices (Ng et al.,1970) perhaps the large injections of L-DOPA were increasing the release of 5-HT which could be maintained in a functional state for a longer period of time because of MAO inhibition. However, Green and Grahame-Smith (1974) found contrasting results in that α-MPT inhibited the hyperactivity due to an MAOI and L-TRY or 5 MeODMT. Brain 5-HT and TRY levels rose, while NA and DA levels were depleted by 75%. The administration of L-DOPA 1 hour after α-MPT almost restored the brain DA concentrations, did not restore brain NA levels but did cause a return of the hyperactivity. An explanation for these differences was proposed by Sloviter and co-workers (1978) who compared gross activity recordings with quantal observation signs. They found rats pretreated with α-MPT

were lethargic and exhibited little spontaneous movement. When these rats received 5MeODMT all signs of the syndrome became evident but the rats were left with fewer gross body movements than were observed with 5MeODMT alone. These conflicting results do not help us to decide whether the syndrome requires an intact DA system for its expression or whether the syndrome caused by an MAOI and L-DOPA is a result of stimulation of DA or 5-HT receptors. Sloviter et al., (1978) found that depletion of catecholamines by α-MPT or 5-HT by either PCPA or 5,7 dihydroxytryptamine did not prevent the syndrome caused by 5-MeODMT. Pretreatment with methysergide but not phenoxybenzamine or pimozide prevented the syndrome caused by 5MeODMT and by an MAOI and L-DOPA. Conversely, 5-HT depletion prevented the syndrome caused by an MAOI and L-DOPA and the behavioural response was restored in PCPA treated rats by 5-HTP. Intraventricular injection of 5-HT or DA caused the behavioural syndrome after MAO inhibition. PCPA pretreatment prevented the syndrome caused by DA but did not prevent that caused by 5-HT.

These results suggest that systemic L-DOPA or intraventricular DA produces the behavioural signs through 5-HT mechanisms: endogenous catecholaminergic mechanisms are not involved directly in either the cause or expression of the behavioural syndrome. This syndrome is of importance as it represents a behavioural index of central serotonergic activity and has been used as such to study the action of various drugs such as chlorpromazine (Grahame-Smith, 1971b), diphenylhydrantion

and chlorimipramine (Green and Grahame-Smith, 1975) and lithium (Grahame-Smith, 1974) which have been used in the treatment of psychiatric disorders in an attempt to elucidate their action on serotonergic neurons and to gain greater knowledge of factors controlling the functional activity of brain 5-HT. For these reasons, this syndrome provided an appropriate model with which to study processes regulating the 24-hour variation in 5-HT, particularly as gross hyperactivity could be measured by activity meters over 24 hours without disturbing the animal's rhythm.

Fig. 39 The effect of tranylcypramine (20mg/kg) on the 24-hour gross activity rhythm of rats.
The intraperitoneal (I.P.) injection 04.00 hours
———tranylcypramine
-----saline

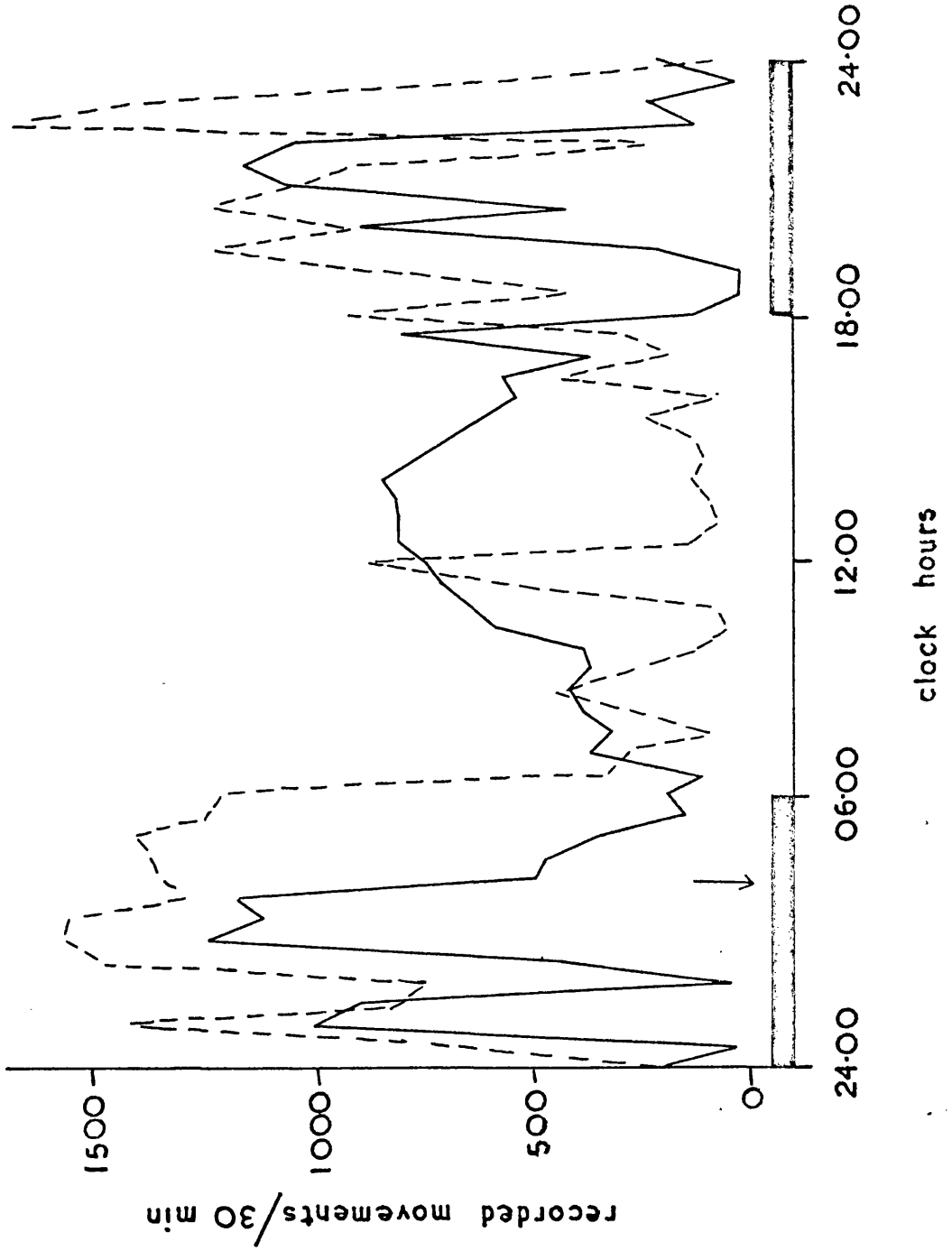

recorded movements/30 min
1500
1000
500
0
24·00
06·00
12·00
18·00
24·00
clock hours

Fig. 39

6.2 Method

90-110g rats were housed in groups of 3 and maintained on the 12 hour light: 12 hour dark cycle previously described (Chapter 2) for 10 days. At the appropriate time of day, an experimental and control group were removed from their controlled environment and unless otherwise stated below were injected with pargyline, followed 30 mins later with either L-TRY (experimental) or 0.9% saline (control). All injections were by the intraperitoneal route and doses varied according to the experiment. The 'Varimex' activity meters were placed in the boxes while the first set of injections were performed to allow time for equilibration. They were linked to a device which recorded the activity monitored by alternate meters every 5 secs. These values were accumulated and printed out every 10 mins. Cages and bedding were weighed as differences affected the sensitivity of the meters. Injections performed in the dark period were carried out using a red light as described in Chapter 2. It should be emphasised that because of the nature of the experiments, it was not possible to observe the animals, nor therefore, to analyse the type of behaviour exhibited: only total activity scores are shown.

Expt. 1 The effect of increasing doses of L-TRY on the hyperactivity produced by an MAOI plus L-TRY over 24 hours. Groups of rats pretreated 30 mins earlier with 20mg/kg tranylcypromine were injected at 16.00 hr (10th hour of light) with either 20, 40 or 60 mg/kg L-TRY or with an equal

Fig. 40 The effect of pargyline (50mg/kg) on the 24-hour gross activity rhythm of rats.
I.P. injection 04.00 hours.
———pargyline
- - - - -saline

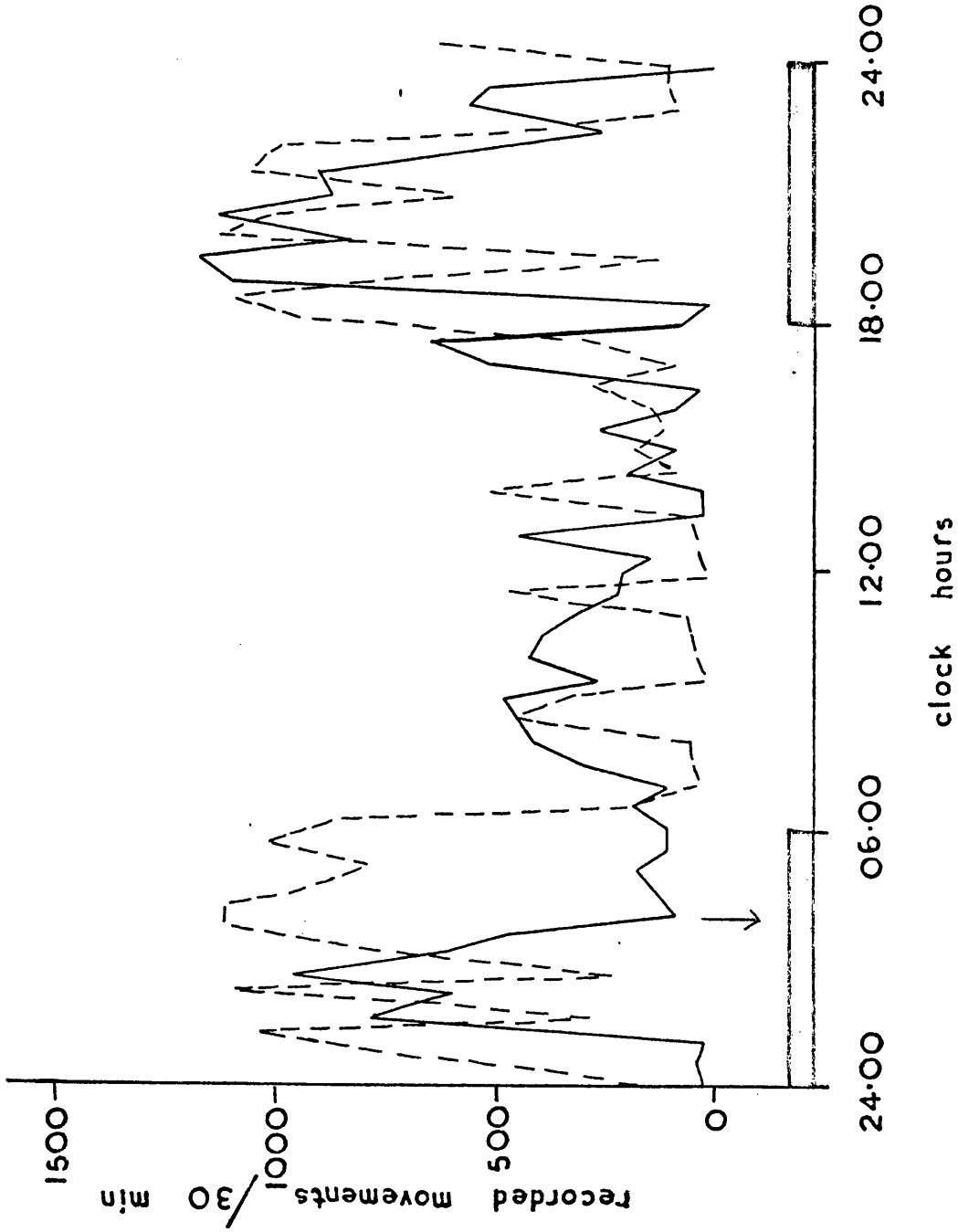

1500
1000
500
0
recorded movements /30 min
24·00
06·00
12·00
18·00
24·00
clock hours

Fig.40

Fig. 41 The effect on gross activity of pargyline (50mg/kg) and L-TRY (50mg/kg) administered at 4 different clock hours.
L-TRY (I.P.) a) 10.00 b) 16.00 c) 22.00 d) 04.00
All pargyline injections (I.P.) 30 min prior to L-TRY dosage.

———exp. (parg./L-TRY)
-----control (parg./saline)

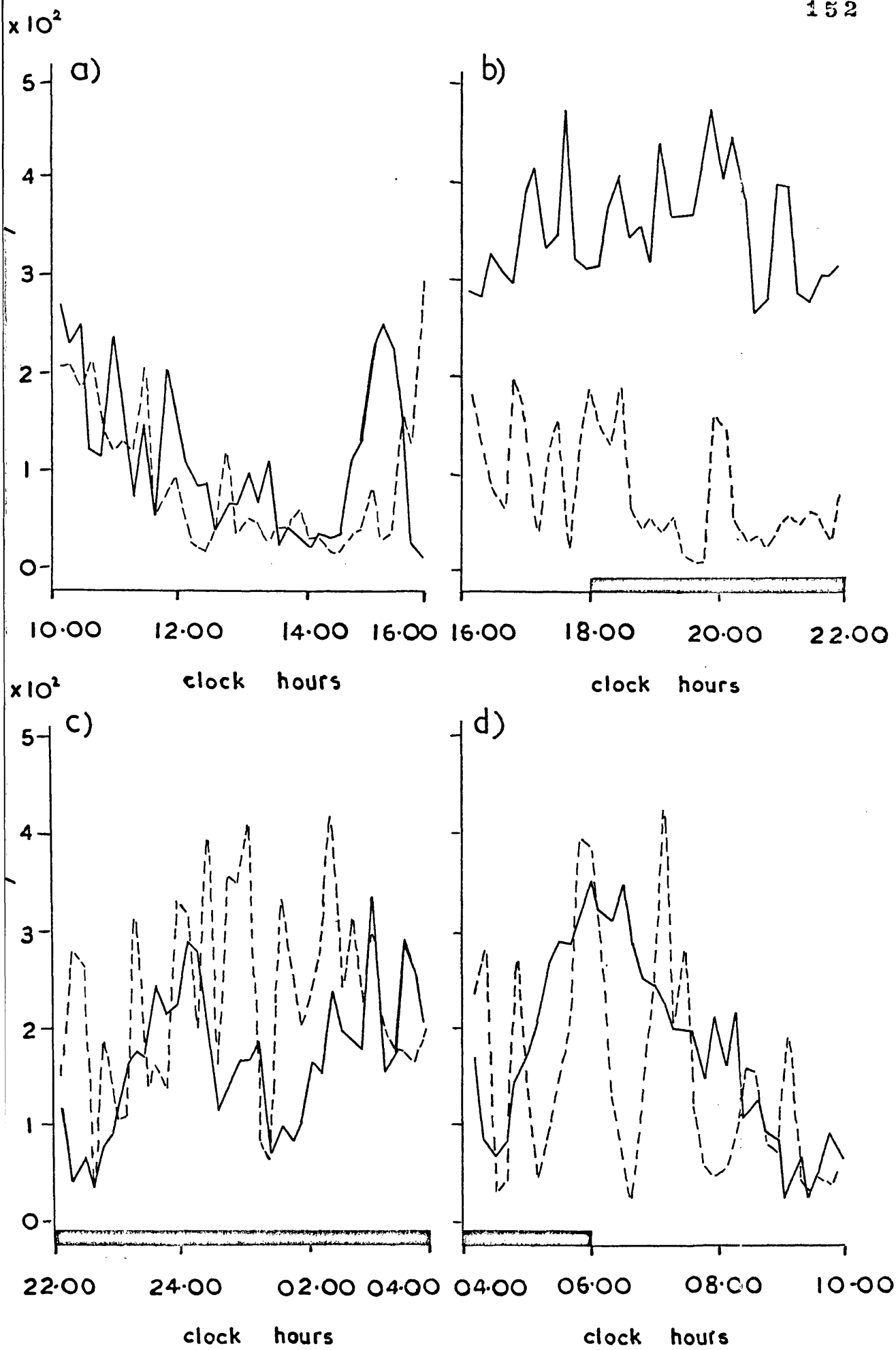

x 10²
a)
b)
5
4
3
2
1
0
10·00
12·00
14·00
16·00
16·00
18·00
20·00
22·00
clock hours
clock hours
x 10²
c)
d)
22·00
24·00
02·00
04·00
04·00
06·00
08·00
10·00
clock hours
clock hours

Fig.41

volume of 0.9% saline. The activity of each group was then monitored for 24 hours.

Expt. 2. A comparison of the effect of paraglyne and trancylcypromine on the activity of rats measured over 24 hours.

Rats were injected at 04.00 hr (10th hour of dark) with either pargyline (50 mg/kg) or tranylcypromine (20 mg/kg) and their activity compared to control groups injected with 0.9% saline.

Expt. 3. The effect of L-TRY plus pargyline injected at 6 hourly intervals (i.e. 22.00, 04.00, 10.00 and 16.00 hours) on activity.

Groups of rats were injected with 50 mg/kg pargyline followed 30 mins later with 50 mg/kg L-TRY or 0.9% saline at the clock hours stated. In each case gross activity was measured for a 6 hour period timed from the L-TRY injection. This was repeated at 16.00 and 04.00 hr using 75 mg/kg pargyline and 50 mg/kg L-TRY.

Expt. 4. The effect of 5 MeODMT injected at 6 hourly intervals on activity (5 MeODMT-hyperactivity).

The above experiment was repeated using 75 mg/kg pargyline and 2.5 and 10 mg/kg 5MeODMT or saline instead of L-TRY. The larger dose (i.e. 10 mg/kg) was only injected at 16.00 and 04.00 hr.

Fig. 42 The effect of pargyline (75 mg/kg) and L-TRY (50 mg/kg) administered at 16.00 hours. Pargyline injected (I.P.) 30 min. prior to L-TRY dosage.
———exp. (pargyline/L-TRY)
------control (pargyline/saline)

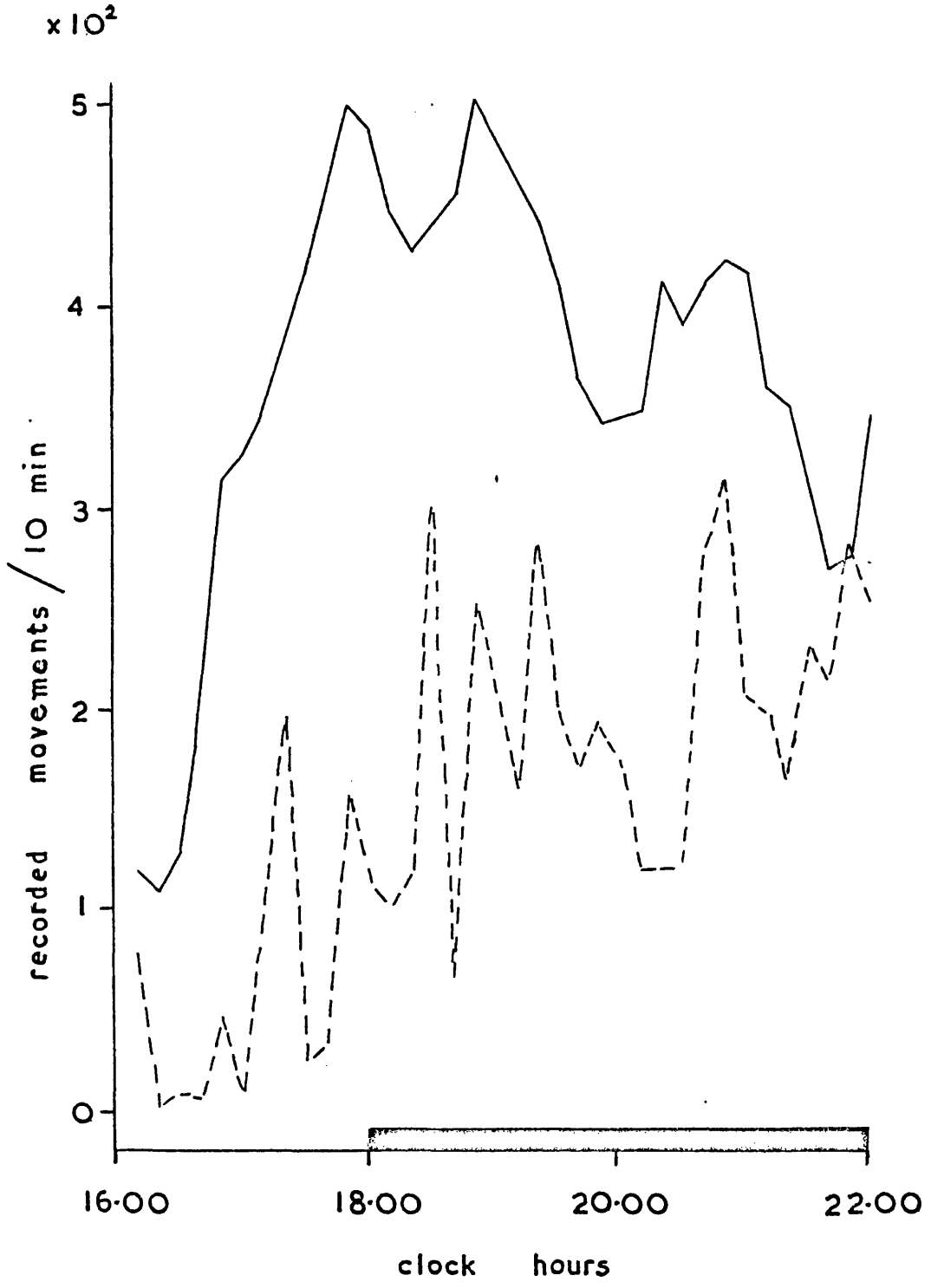

x 10²
5
4
3
2
1
0
recorded movements / 10 min
16·00
18·00
20·00
22·00
clock hours

Fig.42

Expt. 5. The effect of chlorimipramine on the hyperactivity induced by L-TRY plus pargyline (TRY-hyperactivity), injected at 6 hourly intervals.
Groups of rats were injected with 12.5 mg/kg chlorimipramine followed 45 mins later by 75 mg/kg pargyline. 50 mg/kg L-TRY or saline was injected 30 mins after the pargyline dose at 22.00, 04.00, 10.00 and 16.00 hours. The dose of chlorimipramine was then increased to 25 mg/kg and the experiment repeated at 16.00 and 04.00 hours.

Expt. 6. The effect of nomifensine on TRY-hyperactivity.
Groups of rats were injected with 10 or 20 mg/kg of nomifensine followed 45 mins later by 75 mg/kg pargyline. 50 mg/kg L-TRY or saline was injected 30 mins after the pargyline dose at 16.00 and 04.00 hr.

Fig. 43 The effect of pargyline (75 mg/kg) and L-TRY (75mg/kg) administered at 04.00 hours. Pargyline inject (I.P.) 30 min prior to L-TRY dosage.
———exp. (pargyline/L-TRY)
------control (pargyline/saline)

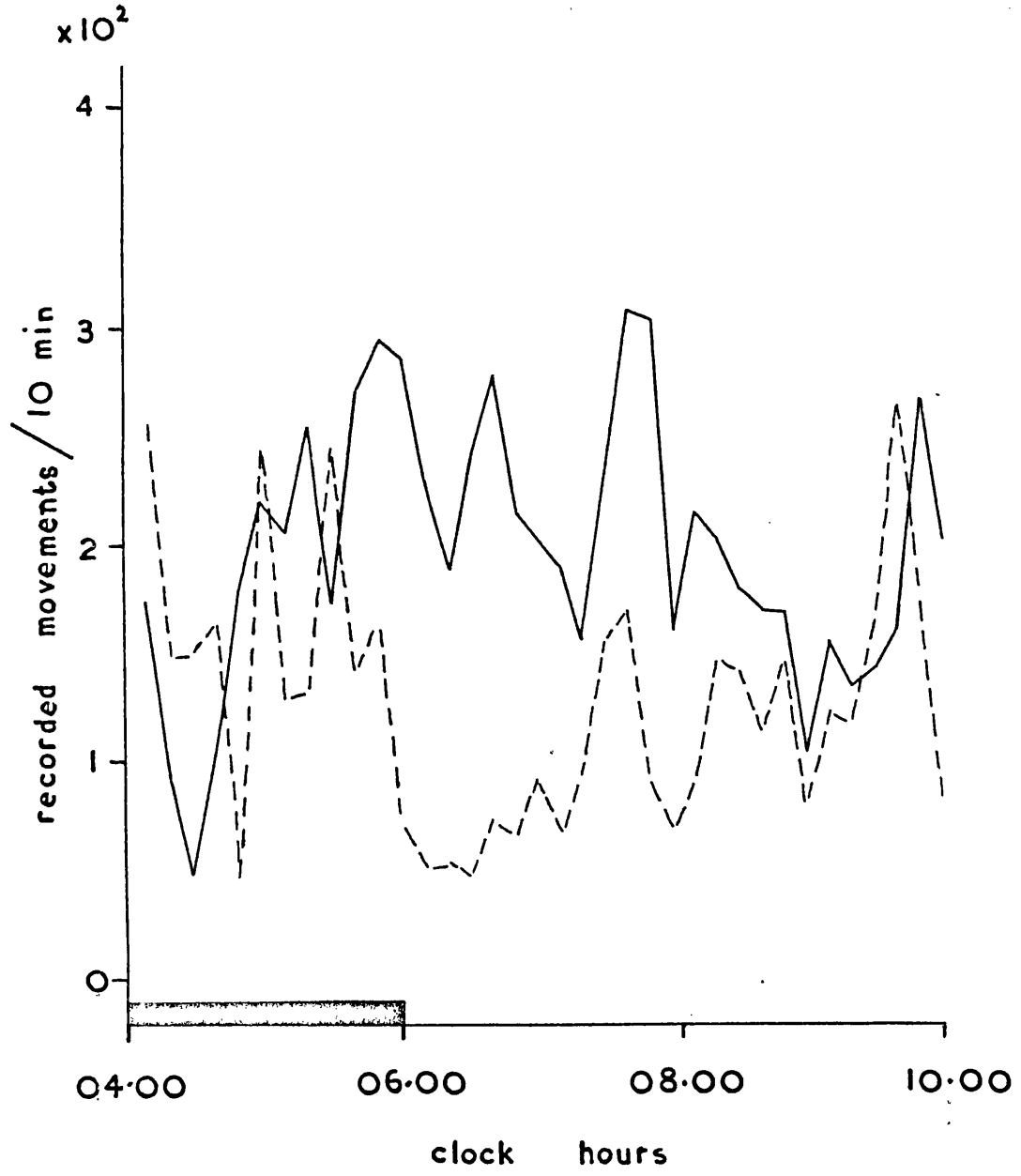

x10²
4
3
2
1
0
recorded movements / 10 min
04·00
06·00
08·00
10·00
clock hours

Fig. 43

6.3 Results and Discussion

In experiment 1 varying doses of L-TRY were used in combination with tranylcypromine to produce the hyperactivity syndrome. These doses were quoted as suitable by Grahame-Smith (1971a); in my experiments, although the doses were effective at producing hyperactivity, they were also lethal. At 60 mg/kg L-TRY, all the experimental rats died, at 40 mg/kg, 66% of the experimental rats died while at 20 mg/kg L-TRY, 33% died. Deaths were not immediate but occurred at least 4 hours after injection presumably due to the effect of excessive hyperpyrexia. It was also noted that the rhythms of the control rats were disrupted. This latter observation led to a comparison of the action of pargyline (50 mg/kg) and tranylcypromine (20 mg/kg) on the normal activity of rats measured over 24 hours. Fig. 39 shows that tranylcypromine produced a certain degree of hyperactivity and disrupted the 24-hour rhythm of the rats. Normally, rats sleep during the light period and very little activity is recorded. The effects of tranylcypromine could be explained by its amphetamine-like effect causing excessive stimulation. For this reason pargyline was subsequently used as the monoamine oxidase inhibitor (Fig. 40).

Doses of pargyline and L-TRY were determined, which produced sub-maximal hyperactivity and which were not lethal. As the hyperactivity syndrome invariably lasted no longer than 5 hours, it was decided to measure activity over 6 hour periods timed from the L-TRY injection rather than over 24 hours. Fig. 40 shows that the control animals' activity

Fig. 44 The effect on gross activity of pargyline (75 mg/kg) and 5 MeODMT (2.5 mg/kg) administered at 4 different clock hours.
5 MeODMT (I.P.) a) 10.00 b) 16.00 c) 22.00 d) 04.00
All pargyline injections (I.P.) 30 min prior to 5 MeODMT dosage.

————exp (pargyline/5 MeODMT)
– – – –control (pargyline/saline)

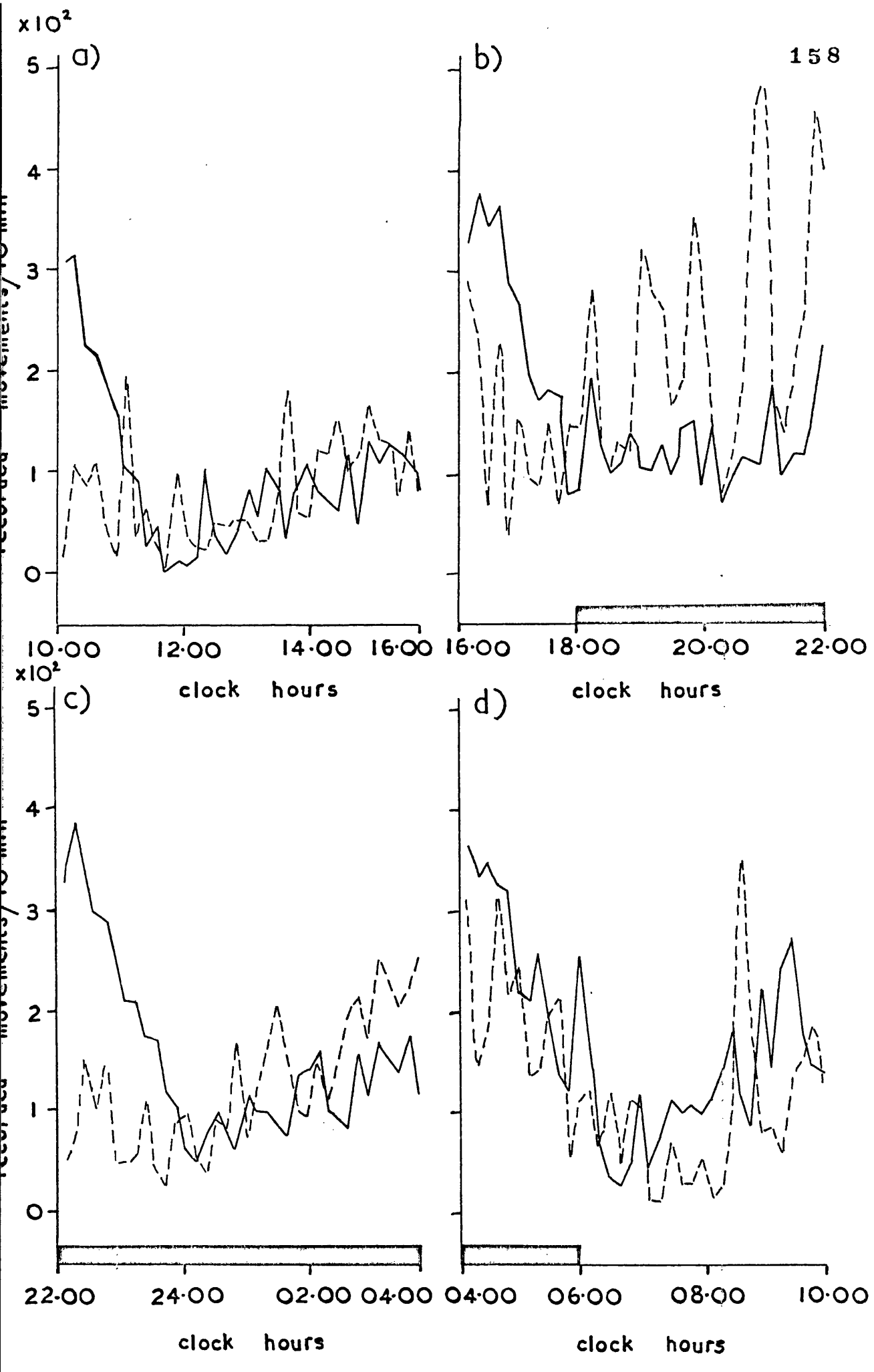

x10²
a)
b)
c)
d)
5
4
3
2
1
0
10·00
12·00
14·00
16·00
16·00
18·00
20·00
22·00
22·00
24·00
02·00
04·00
04·00
06·00
08·00
10·00
clock hours
clock hours
clock hours
clock hours

Fig. 44

Fig. 45 The effect of pargyline (75 mg/kg) and 5 MeODMT (10 mg/kg) administered at 04.00 hour. Pargyline injected (I.P.) 30 min prior to 5 MeODMT dosage.

———exp.(pargyline/5MeODMT)
— — —control (pargyline/saline)

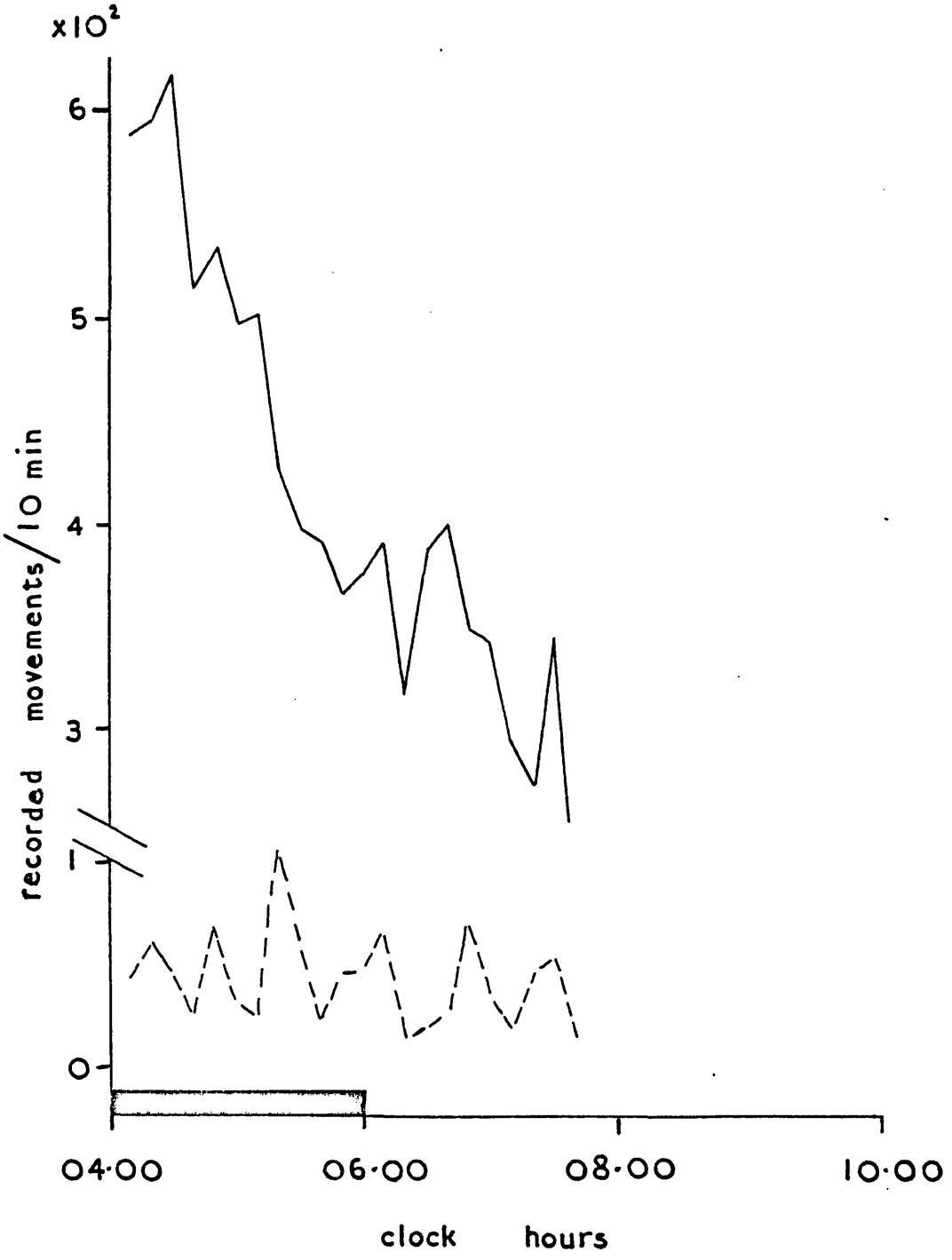

x10²
6
5
4
3
1
0
recorded movements/10 min
04·00
06·00
08·00
10·00
clock hours

Fig. 45

followed a normal cycle with greatest activity occurring during the dark phase which coincided with when the rats ate. Pretreatment with L-TRY plus pargyline did not affect this natural rhythm markedly except when the L-TRY was injected at 16.00 hr (Fig. 41). There was a significant increase in hyperactivity at this time which lasted well into the dark period. This effect was repeated when 75 mg/kg pargyline and 50 mg/kg L-TRY were injected at 16.00 and 04.00 hr (Figs. 42 and 43). Significant hyperactivity was seen at 16.00 hr in contrast to that at 04.00 hr.

The extent of the TRY-hyperactivity induced in this way was compared to that produced by injecting pargyline (75 mg/kg) followed 30 min later by 5 MeODMT (2.5 mg/kg) at the same 4 clock hours. Fig. 44 shows the onset of hyperactivity was rapid and the duration of action was far shorter (approximately 2 hours) than that observed for the TRY-hyperactivity. The level of hyperactivity reached at each clock hour was markedly similar, only the rhythm of the control animals varying. It could be argued that maximal hyperactivity was reached or that a change in behavioural response occurred i.e. perhaps the pattern of the syndrome could change at high doses causing greater stereotyped behaviour which would not necessarily be reflected in an increase in total activity recorded. However, as Figs. 45 and 46 show, when the dose of 5MeODMT was increased to 10 mg/kg, a subsequent rise in hyperactivity was seen.

Fig. 46 The effect of pargyline (75 mg/kg) and 5 MeODMT (10 mg/kg) administered at 16.00 hour. Pargyline injected (I.P.) 30 min prior to 5 MeODMT dosage.

———exp. (pargyline/5MeODMT)
-----control (pargyline/saline)


x 10²
5
4
3
2
1
0
recorded movements / 10 min
16·00
18·00
20·00
22·00
clock hours

Fig.46

Fig. 47 The effect on gross activity of nomifensine (10mg/kg), pargyline (75mg/kg) and L-TRY (50mg/kg) administered at different clock hours.
L-TRY (I.P.) a) 16.00 b) 04.00
Nomifensine and pargyline injected (I.P.) 75 min and 30 min prior to L-TRY, respectively.

———exp. (nomifensine/pargyline/L-TRY)
-----control (nomifensine/pargyline/saline).

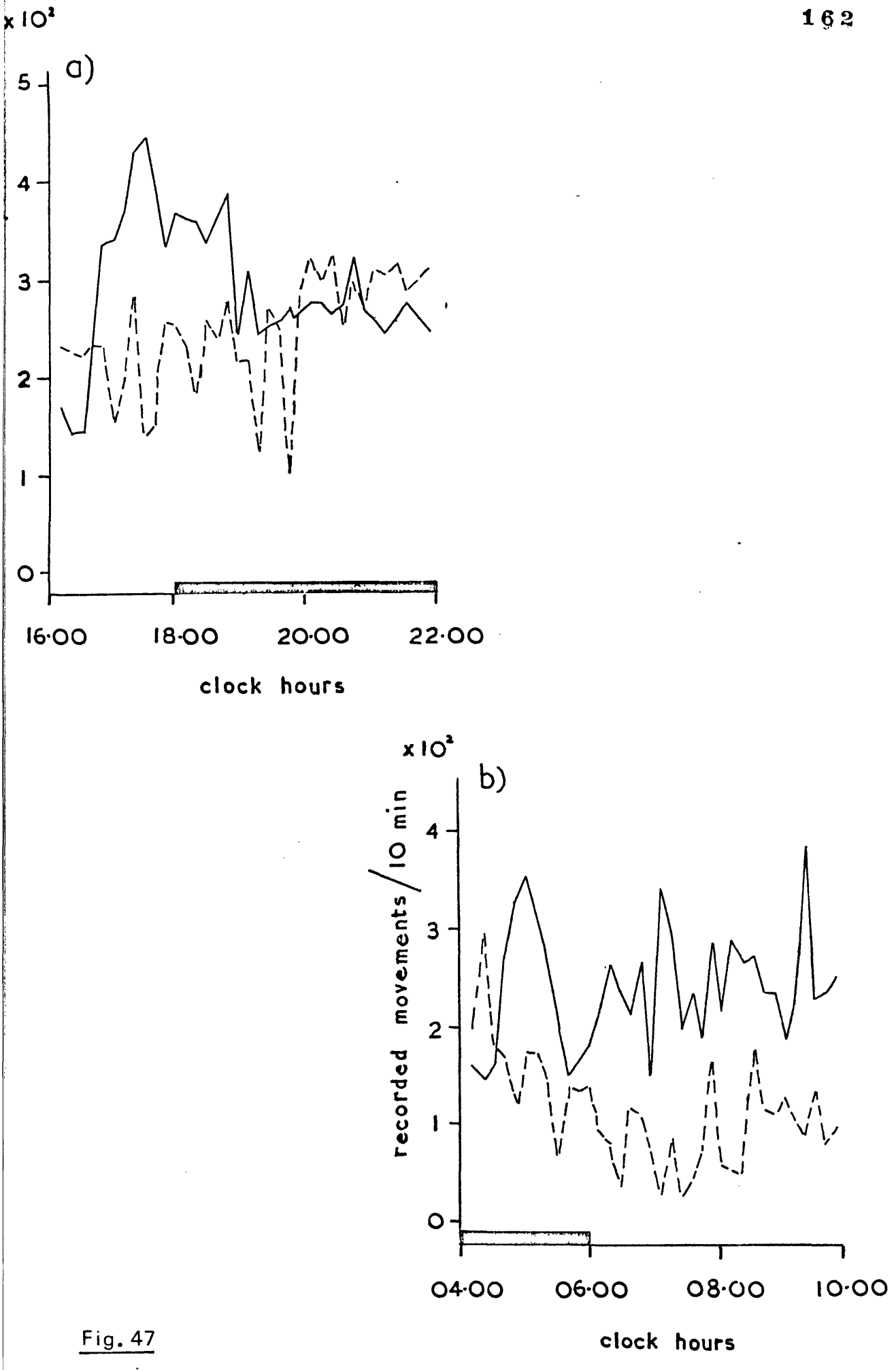

x 10²
a)
5
4
3
2
1
0
16·00
18·00
20·00
22·00
clock hours
x 10²
b)
recorded movements / 10 min
4
3
2
1
0
04·00
06·00
08·00
10·00
clock hours

Fig. 47

These results demonstrate a difference in response to TRY and 5MeODMT. As stated previously, 5 MeODMT is thought to be a post-synaptic receptor agonist of serotonergic neurones (Ahlborg et al., 1968). Therefore, these results again point to the conclusion that variation in response to TRY is due to regulation by pre-synaptic mechanisms rather than changes in post-synaptic receptor sensitivity. Presynaptic regulation could involve synthesis, compartmentation and intraneuronal metabolism or re-uptake. Synthesis regulation has already been considered (Chapters 4 & 5), and previous work in our laboratories had indicated no 24-hour variation in monoamine oxidase activity. It was decided therefore to use this behavioural model to investigate the effects of blocking 5-HT re-uptake of serotonergic neurons using chlorimipramine (Carlsson et al., 1969a, 1969b), on the variation of TRY-hyperactivity. This also gave the opportunity to attempt to assess the role of dopamine in this syndrome by using a newly developed antidepressant drug, nomifensine, which selectively blocks catecholamine (and especially dopamine) re-uptake (Hoffman 1973; Hunt et al., 1974). As Figs. 47 and 48 show, nomifensine (10 and 20 mg/kg) failed to potentiate the TRY-hyperactivity syndrome when measured at 16.00 and 04.00 hr. This is in contrast to the interesting effect seen when chlorimipramine was given together with L-TRY and pargyline. Fig. 49 shows that the TRY-hyperactivity at 16.00 hr was unaltered by chlorimipramine pretreatment whereas the TRY-hyperactivity at the other clock hours increased greatly. The 24-hour variation, seen previously when only L-TRY and pargyline were given, was

Fig. 48 The effect on gross activity of nomifensine (20mg/kg) pargyline (75mg/kg) and L-TRY (50mg/kg) administered at 2 different clock hours.
Experimental detail - refer to Fig. 47.

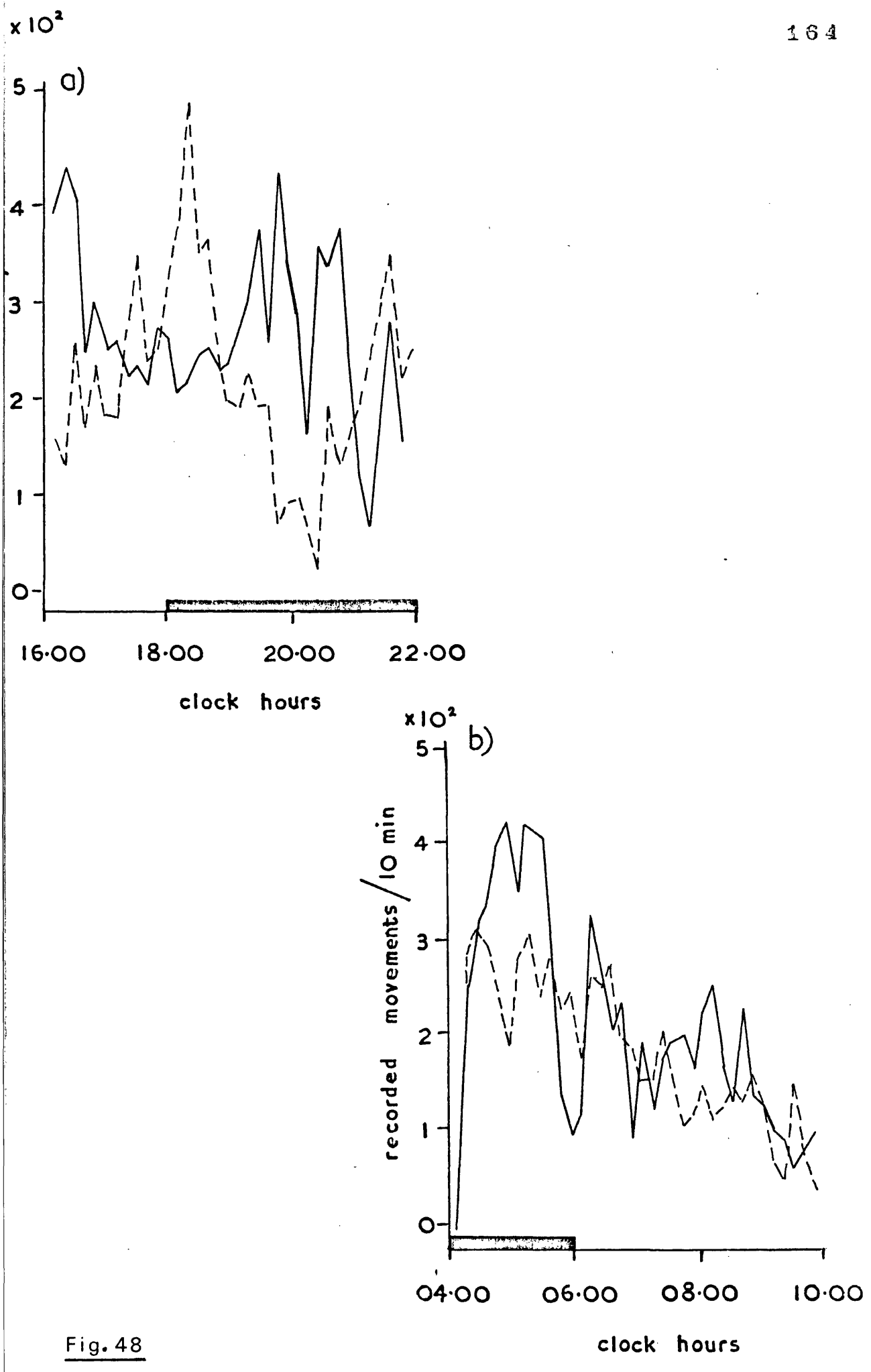

x10²
a)
5
4
3
2
1
0
16·00
18·00
20·00
22·00
clock hours
x10²
b)
5
4
3
2
1
0
recorded movements / 10 min
04·00
06·00
08·00
10·00
clock hours

Fig. 48

Fig. 49 The effect on gross activity of chlorimipramine (12.5mg/kg), pargyline (75mg/kg), L-TRY (50mg/kg) administered at 4 different clock hours.
L-TRY (I.P.) a) 10.00 b) 16.00 c) 22.00 d) 04.00
Chlorimipramine and pargyline injected (I.P.) 75 min and 30 min prior to L-TRY respectively.

———exp. (chlorimipramine/pargyline/L-TRY)
– – –control (chlorimipramine/pargyline/saline)

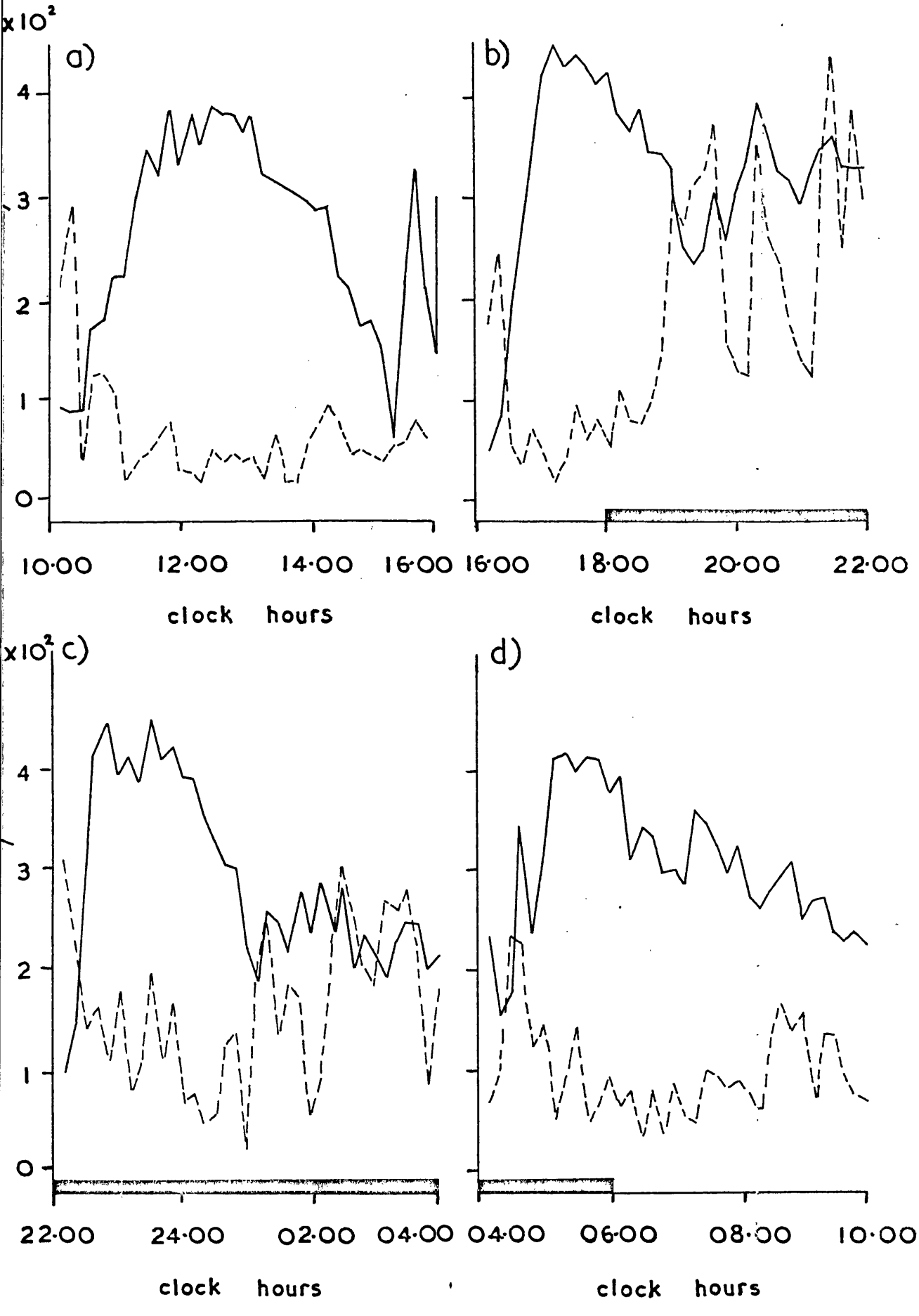

x10²
a)
b)
4
3
2
1
0
10·00
12·00
14·00
16·00
16·00
18·00
20·00
22·00
clock hours
clock hours
x10²
c)
d)
4
3
2
1
0
22·00
24·00
02·00
04·00
04·00
06·00
08·00
10·00
clock hours
clock hours

Fig.49

abolished when the rats were pretreated with chlorimipramine and it could again be argued that in each case a maximal effect was being observed. However, increasing the dose of chlorimipramine to 25 mg/kg increased hyperactivity still further at the 2 clock hours measured (Figs. 50 and 51). Both L-TRY (in the presence of an MAOI) and 5 MeODMT produced a behavioural syndrome of hyperactivity which were distinguishable only by the onset and duration of action. The hyperactivity produced by 5 MeODMT was consistently similar in magnitude and duration at each of the four clock hours measured. As 5 MeODMT is thought to be a post-synaptic receptor agonist, these results indicated that no variation in post-synaptic receptor sensitivity occurred. However, the hyperactivity produced by L-TRY and an MAOI showed a marked variation in that hyperactivity produced at 16.00 hr (10th hour of light) was far more marked than at any of the other clock hours at whichever doses of drug were used. This again led to the conclusion that some pre-synaptic event(s) was responsible for controlling this variation. It was found that the circadian effect of the TRY-hyperactivity was abolished by the re-uptake blocker, chlorimipramine when this was injected prior to pargyline and L-TRY. The hyperactivity was then evident at each clock hour and reached the same level in each instance although this level was not maximal, as increasing the dose of chlorimipramine increased the hyperactivity still further.

Two explanations arise to explain the variation in TRY-hyperactivity seen at different clock hours. The greatest

Fig. 50 The effect on gross activity of chlorimipramine (25mg/kg), pargyline (75mg/kg) and L-TRY (50mg/kg) administered at 10.00 hour.
Experimental detail - refer to Fig. 49.

Fig.50

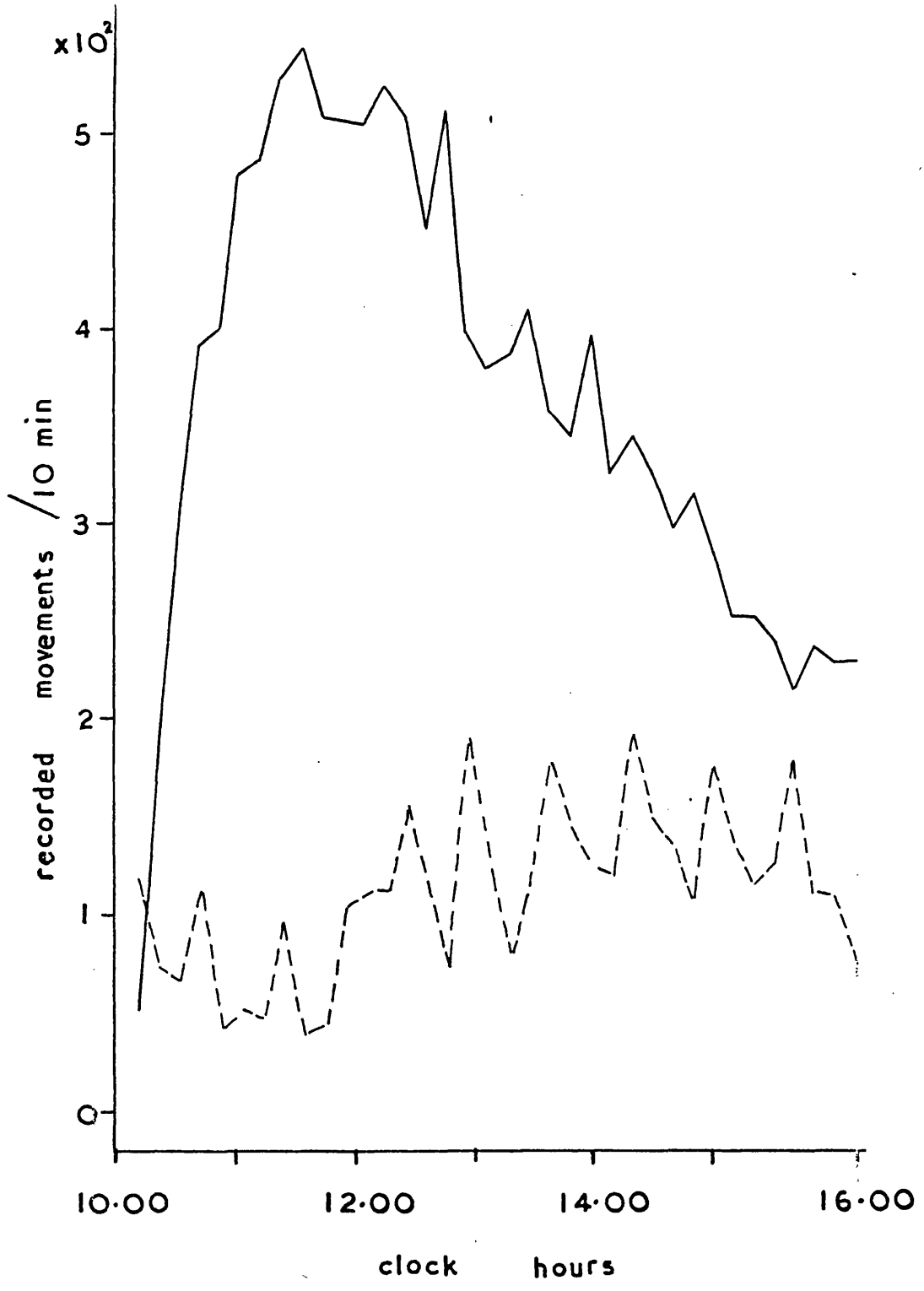
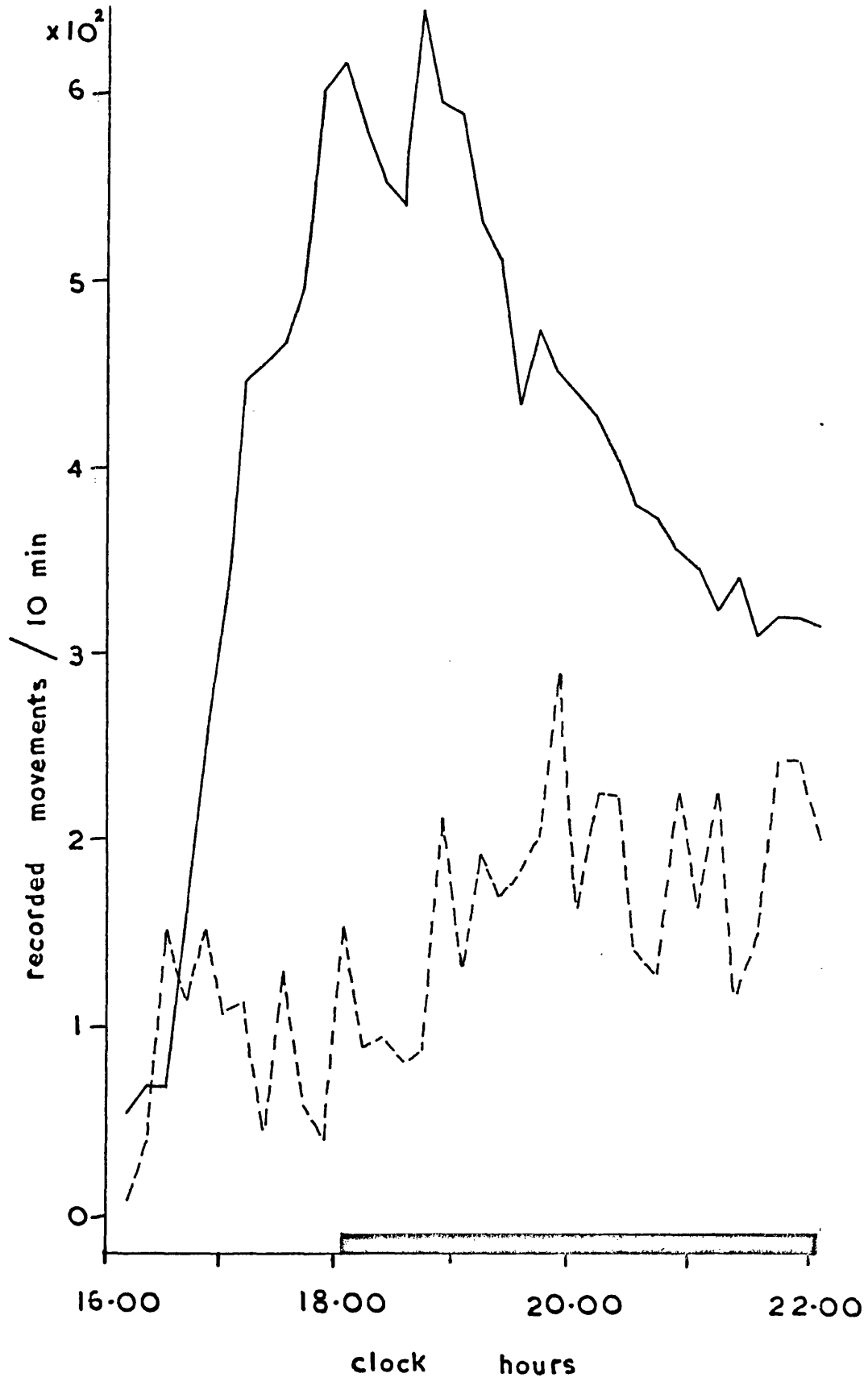

x10²
5
4
3
2
1
0
recorded movements /10 min
10·00
12·00
14·00
16·00
clock hours

Fig. 51 The effect on gross activity of chlorimipramine (25mg/kg), pargyline (75mg/kg) and L-TRY (50mg/kg) administered at 16.00 hour.
Experimental detail - refer to Fig. 49.

Fig. 51


x 10²
6
5
4
3
2
1
0
recorded movements / 10 min
16·00
18·00
20·00
22·00
clock hours

hyperactivity (seen at 16.00 hr) is either due to a release of greater quantities of 5-HT or to a decreased rate of 5-HT re-uptake. The latter could occur if the re-uptake rate was reduced. If re-uptake rate did exhibit variation with clock hour then the action of a re-uptake blocker might be to abolish the effects produced by the variation i.e. the difference in hyperactivity at different clock hours. As stated previously this is in fact the effect chlorimipramine produced; it abolished the variation in TRY-hyperactivity such that the hyperactivity at the other clock hours was increased to the level obtained at 16.00 hours.

These results therefore suggest that the re-uptake of 5-HT into serotonergic neurons may have a regulatory function. This work provides a starting point from which to investigate this phenomenon more closely, particularly by biochemical methods. Wirtz-Justice and Hackman (1973) have shown a circadian variation for the uptake of 5-HT in the hippocampus of the rat brain but were unable to find such a rhythm in other brain regions. The possibility that more 5-HT is available for release at any particular clock hour should also be investigated further. However, this will be considered in more detail in Chapter 7.

CHAPTER SEVEN

Discussion

Discussion

A 24-hour variation in 5-HT levels in the rat brain as discussed in Chapter One has consistently been shown in the rat brain (Dixit and Buckley, 1967; Scheving et al., 1968; Schwartz and Aghajanian, 1969; Okada, 1971; Asano, 1971; Davies and Redfern, 1970). The peak value is reached in the light period when the animals are least active while the lowest levels coincide with the animals' peak activity. The reproducibility of this rhythm leads one to assume that it is under strict control. There is also a 180^{o} phase difference between the 24-hour rhythms exhibited in brain 5-HT concentrations and brain TRY concentrations, the peak values of the latter occurring in the dark period when the maximal food intake of the rats occurs (Le Magnen et al., 1973).

One explanation for the low 5-HT concentration in the dark period is that the turnover of 5-HT is greater. If this were the case, it could be argued that 5-HIAA levels should be raised during the dark phase. Previous findings in our laboratories showed the reverse i.e. the 24-hour variation in 5-HIAA levels closely followed that observed for 5-HT. In addition, the activity of MAO did not vary throughout 24 hours. This strongly suggested that endogenous TRY concentrations do not control the 24-hour rhythm of 5-HT in the rat brain.

From these results it appeared that some pre-synaptic event(s) was controlling the conversion of TRY to 5-HT in the brain to account for the phase difference in their

respective rhythms. When considering neurotransmitter regulation, it is generally assumed that a neuron manufactures only the amount of amine which it requires and that every molecule of metabolite (in this case 5-HIAA) produced equals one molecule of amine released. On this principle the rate of 5-HT synthesis must be precisely 'tuned' to function. Based on this assumption turnover studies *in vivo* measuring the utilization rate of transmitter molecules have been used as a reflection of the rate of synaptic transactions. The most likely sites for biosynthetic 'tuning' are via precursor availability in the brain and within the neuron itself and by regulation of synthetic enzyme activity.

Precursor availability

As stated previously a 180^{o} phase difference between the 24-hour rhythms in brain TRY and 5-HY levels exists which is indicative of factors other than total brain TRY levels controlling 5-HT brain concentrations. However, as discussed in Chapter 3, TRY exhibits the unusual property of binding to serum albumin (McMenamy *et al.*, 1957) which led to the suggestion that it is the plasma free TRY which is the functionally important fraction in determining the availability of circulating TRY to the brain and other tissues (Knott and Curzon, 1972). Perhaps an analogous situation occurs in the brain such that estimation of total brain TRY levels gives misleading results for precursor availability and it is levels of 'free' TRY in the brain which determine the latter.

For these reasons it was decided to measure the concentration of 'free' brain TRY over 24 hours as well as total brain TRY levels. The results obtained (Fig. 20) show that there is a 24-hour variation in 'free' TRY but that the peak values occur in the dark period and the rhythm thus closely follows that for total brain TRY, suggesting neither 'free' nor total brain TRY are directly responsible for the fluctuations consistently measured in 5-HT levels.

In retrospect, it does seem unlikely that a neurotransmitter such as 5-HT, which is possibly involved in such diverse functions as regulation of various types of behaviour, body temperature and sleep, sexual activity, neuroendocrine function would be subject to functional control by dietary intake as discussed in Chapter 3. It is interesting however that all 3 major constituents of the diet i.e. carbohydrate, protein and fat can affect brain 5-HT and possibly suggests a focal role for 5-HT neurons, in allowing the brain to sense peripheral metabolism. It would make some physiological sense if a family of neurons whose function depends on the scarcest amino acid should 'inform' the rest of the brain about the state of the body's metabolism.

TRY uptake

TRY uptake by synaptosomes from the external medium is temperature dependent, inhibited by metabolic inhibitors but not very sensitive to changes in the external concentration of Na^+ or K^+. There appear to be two systems operating,

a low and a high affinity uptake system. Synaptosomes can therefore concentrate TRY. However, the other neutral amino acids inhibit TRY uptake (Grahame-Smith and Parfitt, 1970; Parfitt and Grahame-Smith, 1974). Synaptosomes also show exchange diffusion which is a mechanism whereby a molecule being transported in one direction is linked to efflux and vice versa. It was noted (Grahame-Smith and Parfitt, 1970) that a range of amino acids could exchange with TRY and that the exchange was stereospecific. Clearly therefore, there is no system specifically transporting TRY alone across the neural membrane. The process being shared by several amino acids. Furthermore, TRY uptake does not appear specific to serotonergic neurons. A high affinity transport system was demonstrated in synaptosomal preparations free of serotonergic terminals (Kuhar et al., 1972) and in various cultured cells such as glial cells, fibroblasts and neuroblastoma clones (Bauman et al., 1974). Knapp and Mandell (1972, 1973), investigating the high affinity uptake system, found a disparity between the Vmax of the uptake system and the conversion of TRY, which was found to be at least two orders of magnitude lower (Knapp and Mandell, 1972). Moreover, Kuhar et al., (1972) showed that whereas electrolytic lesion in the median raphe reduced forebrain TRY hydroxylase, it did not significantly reduce the relative velocity of the high affinity TRY uptake system in the forebrain. Both these findings suggest that the high affinity uptake system may not be specific to serotonergic neurons nor indeed, a controlling mechanism.

If a specific uptake mechanism existed for the transport of TRY into serotonergic neurons then the synthesis of 5-HT could be controlled by regulating TRY uptake and thus determining a crucial intracellular concentration of TRY in the vicinity of TRY hydroxylase. However the evidence presented here suggests no such specific mechanism exists. The fact that the TRY transport appears non-specific with respect to serotonergic neurons and selective uptake makes it unlikely that the control of 5-HT synthesis is finely regulated via modulation of TRY uptake into nerve endings. For these reasons, it was decided not to investigate TRY uptake over 24 hours but to concentrate on studying the synthetic enzymes responsible for the conversion of TRY to 5-HT namely TRY hydroxylase and 5-HTP decaboxylase.

The activity of 5-HTP decarboxylase

As stated in Chapter 4 the reason for studying the activity of this enzyme was that preliminary findings in our laboratories had indicated a circadian variation in the activity of a crude enzymatic extract (Hillier and Redfern, 1976). However using a different though equally sensitive method I was unable to repeat this finding. No statistically significant circadian variation was apparent using Fourier's analysis although a significant difference existed between 5-HTP decarboxylase activity measured at 17.00 and 05.00 hours. Although these experiments did not substantiate the preliminary findings it is interesting to note that the rhythm in enzyme activity shown previously was abolished upon purification of the preparation (Hillier and Redfern,1976).

It was shown that the variation was not due to the availability of P5P or to the enzyme being saturated with substrate (Hillier and Redfern,1976). This indicated that the variation observed was not due to an actual change in enzyme activity but to some substance present in the crude extract which was capable of influencing activity. The suggestion was made that competition for the enzyme by different substrates may play a role since a large number of amino acids can be taken up by 5-HT containing neurons. Particular interest was focussed on competition between DOPA and 5-HT. It has been reported for instance that L-DOPA decreases the deamination of 5-HT (Karobath et al., 1972) and that DOPA and 5-HTP compete for the same enzymatic site (Yuwiler et al., 1960). It is also interesting that the 24-hour variation in DA and NA levels are 180° out of phase with that exhibited for 5-HT (Reis and Wurtman,1968; Hillier et al.,1975). Perhaps if levels of the precursors DOPA and 5-HTP vary at different clock hours then the decarboxylation of the one attaining the higher concentration may competitively inhibit the conversion of the other.

I believe, however, it is unlikely that 5-HTP decarboxylase could exert a controlling influence on 5-HT synthesis when the properties of the enzyme TRY hydroxylase are considered. TRY hydroxylase is responsible for the first step in the synthesis of 5-HT i.e. the conversion of TRY to 5-HTP. The maximum velocity of this enzyme is far lower than that of 5-HTP decarboxylase (Ichiyama et al., 1968) and thus

endogenous levels of 5-HTP in the rat brain are negligible (Lindquist et al., 1975) suggesting that TRY hydroxylase is the rate-limiting step in the pathway. It would seem feasible that a far more effective regulation of 5-HT synthesis could be exerted by the activity of TRY hydroxylase especially as evidence discussed in Chapters 3 and 5 indicate it is not saturated with its substrate under normal physiological conditions. For these reasons, the activity of this enzyme was estimated over 24 hours.

The activity of TRY hydroxylase

Using an in vitro method involving $^{14}CO_2$ trapping and a synaptosomal preparation, no 24-hour variation in activity was observed. This suggested that activity of the enzyme was not affected by the availability of the endogenous co-factor or by end product inhibition as regulation by either of these factors would have been evident using an in vitro method. Had a rhythm been in evidence the relative contribution of these factors towards regulation of the enzyme's activity would have required further investigation. The problem associated with using in vitro methods to assay enzyme activity is that the effect of nerve stimulation and hence feedback control via neuronal loops is eliminated. That depolarisation stimulates 5-HT synthesis was originally demonstrated by Anden et al., (1964), who observed an increase in the formation of the transmitter in the isolated spinal cords of mice, subjected to electrical field stimulation.

5-HT synthesis is also accelerated *in vivo* by electrical stimulation of the raphe nuclei (Shield and Eccleston, 1972; Eccleston *et al.*, 1970). Conversely, the interruption of nerve impulse flow in serotonergic neurons induced by transection of the spinal cord in the rat is rapidly followed by a decrease in the rate of TRY hydroxylase (Carlsson *et al.*, 1973). Also the effects on 5-HT synthesis induced by nerve stimulation or by interruption of firing are still observed in rats injected with large quantities of TRY. This indicated that *in vivo* the activity of TRY hydroxylase could be dependent on nerve impulse flow possibly via changes in the affinity of the enzyme for the endogenous cofactor since it was unrelated to the concentration of TRY (Eccleston *et al.*, 1970). From the evidence discussed in Chapter 3 concerning the concept that alterations in plasma 'free' and brain TRY concentrations control 5-HT biosynthesis it would appear that these changes are by no means the only way of functional regulation.

The basic problems inherent in studying enzyme kinetics centrally will not be solved until technical advances allow the estimation of the concentration of neurotransmitter precursors within neurons and also within specific populations of neurons (e.g. the relatively few that actually convert TRY to the related monoamine) and ultimately within specific regions of those neurons (e.g. the perikarya, which uses TRY for protein synthesis and the nerve terminals which are probably the major locus of neurotransmitter synthesis).

These concentrations are probably of major significance in determining the saturation and thus the *in vivo* activities of enzymes that control the utilization of substrates. The true flux of amino acid substrates into the brain, into neurons as a group, into specific neurons and finally into subcellular regions is important as this governs the concentrations within the neurons. Also the extent to which amino acid substrates stored intraneuronally as protein could be used for the synthesis of neurotransmitters may be important. No variation in activity was found for TRY hydroxylase and 5-HTP decarboxylase using *in vitro* methods and the rhythm for 'free' TRY in the brain followed closely that for total TRY. So far, no explanation for the phase difference between the circadian rhythm in TRY brain levels and that of 5-HT levels was apparent. It was then decided to use a behavioural model thought to reflect the action of central serotonergic neurons and to manipulate factors which alter 5-HT synthesis pharmacologically, in the hope that this *in vivo* study might provide more positive results concerning functional regulation.

The behavioural study

To summarise the results from the behavioural work, it was found that TRY-induced hyperactivity showed a circadian effect, the greatest hyperactivity being consistently seen at the 10th hour of light and lasting well into the dark period. In contrast 5 MeODMT-induced hyperactivity showed no variation at any clock hour measured. As 5 MeODMT is regarded as a 5-HT receptor agonist (Ahlborg *et al.*, 1968)

it was suggested that no fluctuation in post-synaptic receptor sensitivity occurred, again indicating that pre-synaptic mechanisms were governing the conversion of TRY to 5-HT. Having found no evidence of regulation via the synthetic enzymes using biochemical *in vitro* studies, the other pre-synaptic factors which could be involved in regulation are compartmentation, intraneuronal metabolism and re-uptake. For the reasons discussed in Chapter 6 the effect of blocking the re-uptake of 5-HT into serotonergic neurons on the variation in TRY-hyperactivity was studied. Chlorimipramine when injected prior to L-TRY and an MAOI abolished the variation in hyperactivity, the latter then reaching the same level at each clock hour. As discussed in Chapter 6 this indicated a variation in re-uptake rate rather than a variation in 5-HT release. Conclusive evidence of this however would require further work particularly biochemical studies. Tricyclic drugs known to block 5-HT uptake (e.g. imipramine and chlorimipramine) inhibit raphe unit firing (Sheard *et al.*, 1972). It has been proposed that these drugs may cause a reduction in 5-HT turnover by means of feedback inhibition of raphe neurons (Corrodi and Fuxe, 1969). Perhaps some feedback mechanism may exist to control the rate of re-uptake over 24 hours.

When considering the regulation of 5-HT biosynthesis, two concepts can be presented as follows:-

1. Any estimate of 5-HT synthesis and turnover relies on the assumption that these processes reflect the activity of brain serotonergic neurons. The implication of this is that a neuron manufactures only the amount of 5-HT which it uses and that every molecule of 5-HIAA produced represents one molecule of 5-HT released.On this principle the rate of 5-HT synthesis must be precisely 'tuned' to function.

2. An alternative possibility is that 5-HT is normally synthesised in excess of functional needs and that the amount of 5-HT actually released is only a proportion of that synthesised. Mechanisms are probably available for the removal of the excess 5-HT synthesised. Storage vesicles are probably not saturated and intraneuronal MAO is available to 'inactivitate' any free 5-HT which reaches it. If the rate of synthesis is in excess of the rate of release and if intraneuronal binding and metabolism operate in a controlled manner. These latter processes might be more important than synthesis in regulating the size of a releasable pool.

In attempting to explain the 180° phase difference between the 24-hour variation in brain TYR and 5-HT levels the results presented in this thesis have led, in fact, to the conclusion that the availability of brain TRY ('free'and total), TRY hydroxylase and 5-HTP decarboxylase activity need not be the fine controls by which the amounts of 5-HT are available for functional activity. The behavioural studies

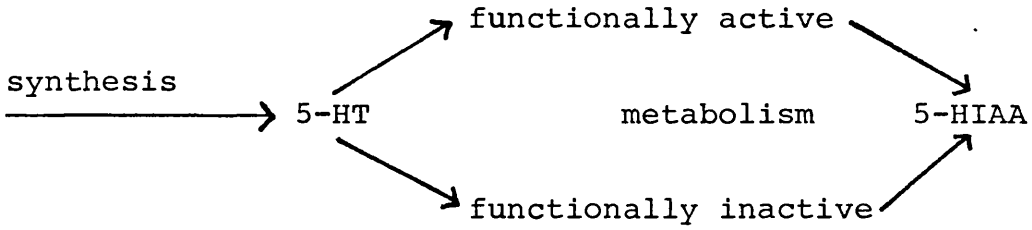
indicate again however that the variation in activity is controlled by a pre-synaptic rather than a post-synaptic event. These conclusions indicate (although untested) that maybe it is intraneuronal binding and metabolism together with re-uptake that are important, and therefore support the second theory proposed. Perhaps the most relevant finding, supporting this theory is that when TRY is given alone to rats, no gross behavioural change occurs yet when TRY is given to rats pretreated with an MAOI, a hyperactivity syndrome is seen. These two situations differ only in regard to the inhibition of MAO. With TRY alone the 5-HT synthesised does not exert functional activity i.e. behavioural change because of the binding capacity of intraneuronal vesicles and the activity of MAO. With TRY plus a MAOI, 5-HT is being synthesised at an equivalent rate, presumably saturating storage sites and 'spilling over' into functional activity (Grahame-Smith 1971b). Further evidence to support this theory is that lithium can produce a 70% increase in 5-HT turnover but no overt behavioural changes (Grahame-Smith and Green, 1974). The observation of animal behaviour is a relatively crude index of drug action and this makes extrapolation from animal neuropharmacology to changes in the human mind, such as mood, extremely difficult. Thus, a failure to observe a behavioural change in animals is no proof that a change in mental function would not have occurred in man. Nevertheless there is some clinical evidence to suggest that large plasma TRY changes can occur acutely with little effect on mood.

Greenwood et al., (1974) infused intravenous TRY up to a dose of 100 mg/kg producing a forty-fold increase in free TRY and an eight fold increase in the bound form. Few differences were observed in objective psychological tests although there were minor EEG changes and some changes in mood self-rating scales. Green et al., (1978) gave L-TRY (50 mg/kg) orally as part of a metabolic study and produced a seven fold increase in total TRY levels in plasma. The 24 subjects reported no subjective changes in mood or ability to perform normal tasks.

However, there is evidence that giving TRY alone (Broadhurst, 1970) or with an MAOI (Coppen et al., 1963; Pare, 1963; Glassman and Platman, 1969) is effective in the treatment of depression. It has to be considered that the anti-depressant effect of TRY could be due to the formation in the brain of compounds other than 5-HT. For instance tryptamine (Saavedra and Axelrod, 1973; Marsden and Curson, 1974) and 5 methoxytryptamine (Green et al., 1973; Green et al., 1975), both of which have been found in the brain and produce behavioural effects.

If the control of the amount of 5-HT available for activity is exerted upon vesicular binding and oxidative deamination one specific problem arises when one attempts to distinguish between functionally active and functionally inactive 5-HT. 5-HT released into the synaptic cleft, re-uptaken and intraneuronally metabolised has exerted a post-synaptic

effect i.e. it has been functionally active. If 5-HT is produced in excess of needs and the excess is controlled by storage and intraneuronal metabolism, then the latter is functionally inactive. This places doubts on turnover studies reliant on 5-HIAA estimations as reflections of the functional state of 5-HT dependent neurons as the 5-HIAA could be derived from 2 pools, one from released 5-HT and the other from non-released 5-HT oxidatively deaminated within the neuron.



This also highlights the hazards of interpreting changes in CSF 5-HIAA levels associated with affective disorders, in terms of the functional activity of 5-HT and its primary aetiological involvement in psychiatric disorders. Perhaps these ideas however can explain why 5-HT levels appear so susceptible in particular instances to plasma TRY fluctuations. If 5-HT is synthesised in excess then as long as MAO is intact, fluctuations in 5-HT levels will in no way affect serotonergic function.

The behavioural studies indicate a diurnal variation in re-uptake. It is possible that this could affect the magnitude of the response to 5-HT at the post-synaptic receptors and would therefore be affecting functional 5-HT once it is released. However, this provides no insight into factors controlling functional 5-HT intraneuronally i.e. intraneuronal binding and MAO activity. The effect of the latter systems in regulating the 24-hour variation in 5-HT levels will be far more difficult to establish. One difficulty arises from the inability to inhibit intra- and extraneuronal MAO activity differentially. Another problem is that the control of 5-HT could depend upon the relative activities of the different forms of MAO. MAO has been shown to exist in 2 forms, 'Type A' and 'Type B' (Youdim and Sandler, 1967) and the latter is ineffective in metabolising 5-HT. If these forms do indeed metabolise monoamines differently in vivo, changes in activity could occur in one form, thus controlling a particular amine concentration without altering others. These changes would be masked if total MAO activity alone were measured. For these reasons, an interesting study for further work would be the effect of specific 'type A' and 'type B' MAO inhibitors in combination with L-TRY to produce hyperactivity, using respectively, clorgyline (Johnston, 1968) and deprenil (Sandler and Youdim, 1974; Houslay and Tipton, 1974). Also the effect of disrupting vesicular binding on the TRY-hyperactivity syndrome over 24 hours and a comparison of the action of other 5-HT re-uptake inhibitors e.g. Lilly

110140 (Fuller et al., 1974) with chlorimipramine to see if comparable results are obtained would be valuable.

If one accepts the concepts presented here that organisation of intraneuronal pools and controlled intraneuronal metabolism, rather than the rates of synthesis, are all important in controlling functionally active 5-HT one must inevitably ask the question, "What is the relevance of the 24-hour variation of 5-HT levels in the rat brain?" Until we are able to estimate whether the levels measured are actually released into the synaptic cleft, the question must remain unanswered.

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